THE ROLE OF THE FUSED TOES LOCUS CONTAINING IROQUOIS HOMEOBOX GENE 3, 5, AND 6 AND FTM, FTO, AND FTS DURING OVARY AND TESTIS DEVELOPMENT IN MICE

BY

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DISSERTATION

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ABSTRACT

These studies were conducted for a greater understanding of the function of the Fused toes (*Ft*) locus, which includes six genes: homeobox genes: *Irx3*, *Irx5* and *Irx6* and three other genes: *Fto*, *Fts*, and *Ftm*, during male and female gonad development.

Ft mutant mice are missing *Ft* locus genes whose function is unknown during male and female gonad development. These six missing genes show stage and sex specific expression patterns during sex determination and differentiation in mouse gonads. *Irx3* and *Irx5*, which are the homeobox genes, and *Ftm* and *Fts* are highly expressed in ovaries from the stage of early gonad development. However, *Ftm* shows a dramatic increase in adult testes. This dimorphic expression pattern gave us the following central hypothesis. The *Ft* locus is a new potential gene cluster essential for normal ovarian and testis development. To elucidate this main hypothesis, I have conducted three serial studies 1) to investigate the function of the *Ft* locus in developing female gonads, 2) to examine the function of the *Ft* locus in developing male gonads, 3) to determine the functional roles of *Irx3* and *Irx5* during developing male and female gonads.

In the first and second studies, *Ft* mutant mice were used to investigate functional roles of *Ft* locus. In the absence of the the *Ft* locus, the primordial germ cells (PGC) develop normally, however, many PGC fail to migrate to the genital ridge and fail to proliferate once they reach the genital ridge. Failure of the PGC migration and proliferation causes a significant reduction in the number of germ cells. In addition, the *Ft* mutant ovaries revealed a disruption in their follicle formation, as most follicles were blocked before secondary follicle formation. Also, a lower expression level of Foxl2, which is known as a key gene for normal follicle development, was observed in the *Ft* mutant granulosa cells.

My second study was to investigate male testis development in the absence of the *Ft* locus. In the absence of the *Ft* locus, many PGC failed to arrive at the genital ridge and to proliferate described in the female gonad. Otherwise, fetal testis development was largely normal. After birth, however, I observed a failure of Sertoli cell maturation in *Ft* mutant testes. The *Ft* mutant testis showed a low level of MIS expression in the Sertoli cells and a partial failure of germ cell movement toward the basement membrane.

The last study in the series was to begin to determine the function of *Irx3* and *Irx5* during male and female gonad development. To investigate whether these two genes are necessary for testis or ovary development, I generated conventional and conditional *Irx3/5* double knockout mice. Interestingly, the absence of *Irx3/5* during the early stage (~E13.5) did not have any effect on ovary and/or testis development but resulted in a loss of germ cells and a partial disruption of granulosa cell formation in postnatal day 0 (P0) stage. However, there were no difficulties in testis development.

In summary, this thesis presents evidence that the *Ft* locus plays a critical role during embryonic and postnatal ovarian and testis development. In addition, we present evidence to propose that the proper expression patterns of six genes within the *Ft* locus are necessary for normal ovarian and testis development through maintaining communication between somatic and germ cells.

To my wife Youngha and son Jinhyuk

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LIST OF ABBREVIATIONS

Blimp1: B-lymphocyte-induced maturation protein-1

BMP: Bone morphogenetic protein

CYP17: 17-alpha-hydroxylase

DES: Diethylstilbestrol

DMSO: Dimethyl sulfoxide

DV: Dorsal-ventral

E: Embryonic day

E2: Estradiol

EGFP: Enhanced green fluorescenent protein

EMX2: Empty-spiracles homeobox gene 2

EXE: Extraembryonic ectoderm

FGF9: Fibroblast growth factor 9

FIGLA: Factor in the germline alpha

Foxl2: Forkhead box L2

Ftm: Fantom

Fto: Fasto

Fts: Fused toes

GCNA: Germ cell nuclear antigen

GDNF: Glial cell line-derived neurotrophic factor

HIF: Hypoxia-inducible factor

HMG: High mobility group

IrxB: Iroquois homeobox B

Kit: Kit receptor

Kitl: Kit lignad

Lhx9: Lim-like homeobox gene 9

MIS: Müllerian inhibiting substance

MOFs: Multiple oocyte follicle

NOBOX: Newborn ovary homeobox gene

P: Postnatal day

PFA: Paraformaldehyde

PGC: Primordial germ cells

p-HHH3: Phosphorylated histone H3

PM: Peritubular myoid

RA: Retinoic acid

Rspo-1: R-spondin-1

SCF: Stem cell factor

SDF1: Stromal cell-derived factor 1

SSEA-1: Stage-specific antigen-1

SF1: Steroidogenic factor 1

Sox9: Sry-related HMG box gene 9

Sry: Sex determining region of the Y chromosome

StAR: Steroidogenic acute regulatory protein

Stra8: Stimulated by retinoic acid gene 8

TCF: T cell factor

TGF: Transforming growth factor

VE: Visceral endoderm

Wt1: Wilms tumor suppressor 1

CHAPTER 1

LITERATURE REVIEW

This thesis focuses on the effects of the Fused Toes (*Ft*) locus, which contains six genes: *Irx3*, *Irx5*, *Irx6*, *Ftm*, *Fto*, and *Fts*, during gonadal development in mice. Specifically, these studies describe ovarian and testis development during fetal and on through early postnatal stages in *Ft* mutant mice. To understand the context, I briefly describe the process of ovarian and testis development in mice and include information regarding the *Ft* mutant mouse.

1.1: OVARY vs. TESTIS

The ovary and the testis both develop from a single primordium. For this reason, the gonadal primordium is called "the bipotential gonad'. These bipotential gonads can develop into either sex depending on specific factors. The gonad is therefore a unique system for the study of cell fate decisions and organogenesis [1].

What functions does an ovary have? The ovary has two main functions: 1) the production of steroid hormones and 2) the generation of mature oocytes that are capable of being fertilized and develop into an embryo. The functions of the testis are identical to the ovary. It also produces and secretes steroid hormones to support spermatogenesis and produces the male germ cells that are necessary for formation of the conceptus.

Both ovaries and testes consist of two types of cells which are the germ cells that form germ cell lineage and somatic cells that make up the somatic cell lineage. The interactions between germ cells and somatic cells are essential to form normal ovaries and testes. These two

kinds of cell lineages are unique during gonadal development. Therefore, I will describe the development of the germ cell and somatic cell lineages.

1.1.1: Germ cell lineages

1.1.1.1: Germ cell specification

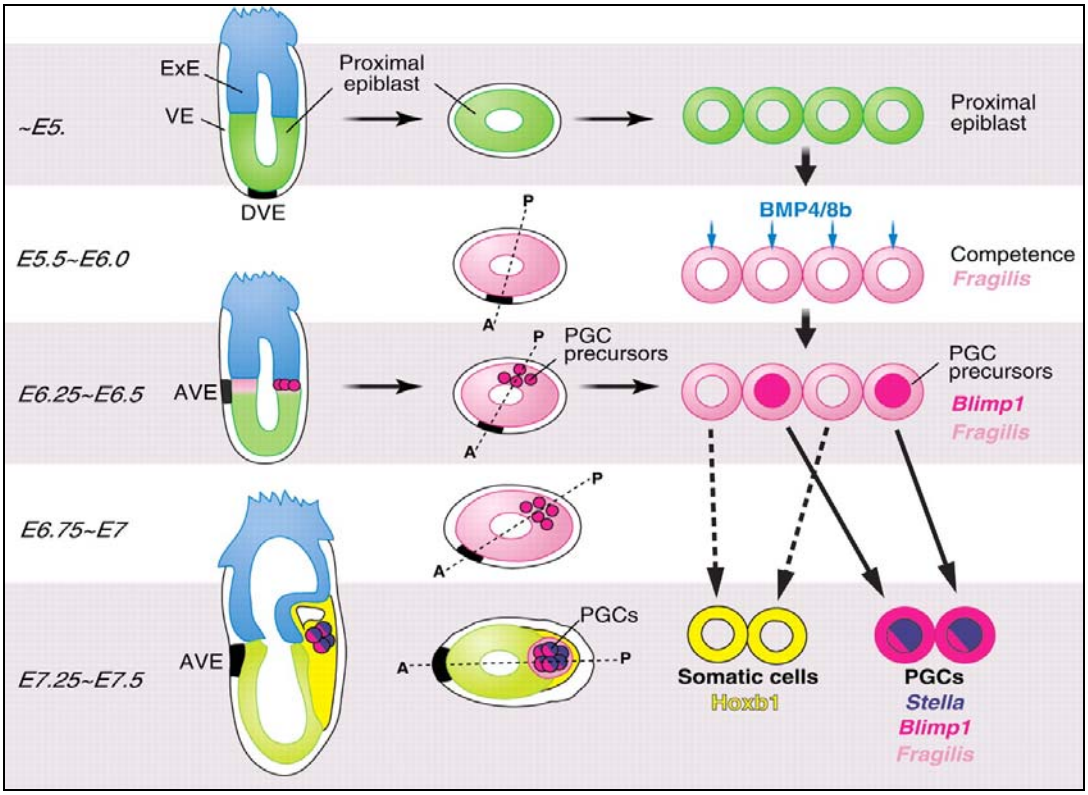
Specification of germ cell fate is fundamental in development and heredity. In mice, and presumably in all mammals, germ cell fate is not an inherited trait from the egg, but is induced in pluripotent epiblast cells by signaling molecules (Fig. 1.1) [2]. Bone morphogenetic protein 4 (Bmp4) and Bmp8b, members of the TGF beta superfamily, are produced by the extraembryonic ectoderm (EXE). Targeted inactivation of either gene results in a failure to form primordial germ cells (PGC) [3, 4]. In addition, Bmp2, a close relative to Bmp4, is expressed primarily in the visceral endoderm (VE), especially in the junctional area between the EXE and epiblast, and also plays a role in the establishment of the germ cell lineage. The number of PGC is significantly reduced by targeted inactivation of Bmp2 [5]. Therefore, at least three Bmps, Bmp4 and Bmp8b from EXE and Bmp2 from VE, are necessary for the proper establishment of PGCs.

Several Smad proteins (Smad1, 5, and 8) serve as intracellular signal transducers for Bmps. Although the expression of Smad 8 was not detectable in epiblasts at any stages, Smad1 and Smad5 are expressed widely in the epiblast during gastrulation. Loss of Smad1 and Smad5 causes severe reduction in the number of PGC [6, 7].

Additionally, Expression of the region-specific homeobox genes including *Hoxa1*, *Hoxb1*, *Lim1*, and *Evx1* which are highly up-regulated in somatic mesodermal neighborly cells is specifically repressed in PGCs [8-12]. The strong expression of *fragilis* causes *Hoxb1* repression, followed by expression of *stella* within PGC [10]. The repression of Hox genes in PGC is

proposed to be one of the mechanisms by which the PGC escape from the somatic fate and retain their pluripotency [13]. Another gene, *Blimp1* (B-lymphocyte-induced maturation protein-1, also known as *Prdm1*) is also proposed to be a key factor for germ cell specification [14-16]. In *Blimp1* mutation, PGC specification seems to be halted at a very early stage with small numbers of PGC-like cells. In addition, these PGC-like cells fail to repress Hox genes and activate some of the PGC-specific genes such as *stella* [16].

Figure 1.1: Early primordial germ cell specification from E5.0 to E7.5 in mice.
(Adopted from Katsuhiko Hayashi et al. 2007)
The proximal epiblast cells respond to signals from the extraembryonic tissues, which induce expression of *fragilis* in the epiblast, and of *Blimp1* in the PGC precursor cells at one end of the short axis before gastrulation. After gastrulation, the PGC precursors locate to the posterior proximal region, where they undergo specification to form the founder population of *Stella*-positive PGC.



1.1.1.2: Migration and proliferation of PGC

A cluster of about 50 specified PGC in the extraembryonic mesoderm, posterior to the primitive streak, begin to individually migrate toward the future genital ridges starting at around E7.5 [17]. By E8.5, PGC are localized around the hindgut diverticulum, and by E9.0 they become incorporated into the hindgut epithelium [17]. PGC exit from the dorsal aspect of the hindgut between E9.0 and E9.5, split into two streams of individual cells, and migrate laterally across the dorsal body wall into the developing genital ridges [18]. At E10.5, PGC located close to the genital ridges continue to migrate, singly or in clusters, into the genital ridges. The PGC numbers increase from less than 100 at E8.5 to 3,000 at E11.5, indicating a cell cycle length of approximately 16 hours [19].

The precise molecular mechanisms governing the PGC migration process are unclear but the effects on the viability or behavior of PGC during their migration have been studied through the use of a number of mutations [2]. *Steel* factor (also known as stem cell factor, kit ligand or mast cell growth factor) and W loci (also known as cell surface tyrosine kinase receptor, c-kit) are critical for the migration, proliferation, and/or survival of PGC. In c-kit mutants, PGC specification seems apparently normal but the numbers of PGC do not increase after E8.5 in their migration period [20, 21]. In addition, Steel factor is necessary for PGC survival throughout their migration, from the time of formation to the time of colonization of the gonads [18].

The POU domain transcription factor Oct4 is another necessary factor for the survival of PGC. Germ cell specific deletion of Oct4 impairs PGC maintenance leading to high levels of apoptosis before germ cell colonization into the genital ridge [22]. The mechanism by which loss of Oct4 causes a reduced number of PGC remains to be determined.

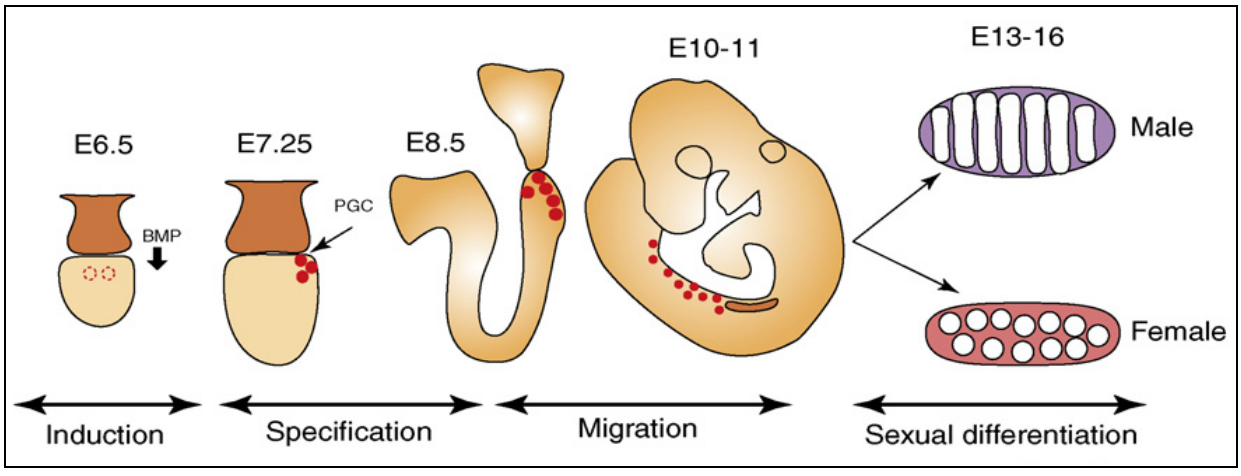
The *Nanos* gene, which encodes an RNA binding protein, was first identified as a maternal effect gene in Drosophila [23]. In the absence of maternal Nanos, PGC failed to migrate into the gonad and did not become functional germ cells [24, 25]. In the mouse, Nanos3 mutants undergo normal PGC specification but the number of PGC is reduced after E8.0, and eventually all the PGC are lost [26]. This mechanism of Nanos3 function during PGC migration also remains to be determined.

TIAR, an RNA recognition motif/ribonucleoprotein-type RNA-binding protein [27], is also essential for the survival of PGC [28]. TIAR may affect the stability of mRNA encoding growth factors such as steel factor, LIF, basic fibroblast growth factor, interleukin 4, or their respective receptors, all of which are critical for the survival and proliferation of PGC [29-32]. However, how TIAR promotes the survival of PGC is unknown. HIF-2alpha (Hypoxia-inducible factor-2alpha) also plays a critical role for PGC specification and/or the survival of PGC. In HIF-2alpha mutant mice, the numbers of PGC are severely reduced as early as E8.0 because they fail to up-regulate Oct4 [33].

PGC specification and migration are critical processes for normal ovary and testis development. Both male and female PGC show the same fate until arriving in the gonad which is their final destination. In both sexes, mitotic proliferation of germ cells continues for 2-3 days after entry into the genital ridge [34]. Germ cells show dramatic changes of their phenotype at the time of colonization in the genital ridge. In germ cells, high alkaline phosphatase activity continues but expression of stage-specific antigen 1 (SSEA-1) is down-regulated by about E12.5, while new antigens appear including germ cell nuclear antigen 1 (GCNA) [35], and mouse vasa homologue [36]. At around E13.5, both genders of germ cells are distinguishable from one another. Female germ cells enter meiotic prophase and pass through leptotene, zygotene and

pachytene before arresting in diplotene around the time of birth [37]. Meanwhile male germ cells undergo mitotic arrest and remain in G_1/G_0 until after birth [38]. After arriving in the gonads, the fate of both male and female germ cells is dependent on interactions with the gender specific somatic component of the developing gonad, not on the chromosomal sex of the germ cells [39]. The signals from adjacent somatic cells direct the differentiation of germ cells within the embryonic gonads [40].

Figure 1.2: Illustration depicting germ cell development in mice.
(Adapted from Yumiko Saga. 2008)
Primordial germ cells (PGC) are induced in the epiblast by BMP signaling at E5.5–E6.5. PGC are specified through the function of Blimp1 by E7.25. PGC are protected from apoptotic signals during their migration via Kit-mediated signaling, and through the functions of Nanos3 and Dead end1. Once PGC reach and enter the genital ridge at E11.5, they differentiate according to the somatic sex of the embryo. Nanos2 promotes the male germ cell fate by suppressing the female fate. The locations of the PGC are indicated by red circles.



1.1.2: Somatic cell lineages

Between E10.0 and E11.0 in the mouse gonad, while most germ cells are entering into gonads, the somatic cells of both sexes are positive for several genes that are crucial for the initial formation of the bipotential genital ridge including the empty-spiracles homeobox gene 2 (EMX2) [41], Wilms tumor suppressor 1 (Wt1) [42], Lim-like homeobox gene 9 (Lhx9) [43], and Steroidogenic factor 1 (SF1) [44].

The homeobox gene *Emx2* is expressed in the mesonephric ducts, tubules and coelomic epithelial parts of the urogenital system [45]. In $Emx2^{-/-}$ mutants, the migration of PGC occurs normally, but they result in sex-independent absence of the gonads and genital tracts such as Müllerian duct and Wolffian duct [41]. Its role in the development of early gonad is unclear.

Wilms tumor suppressor 1, a zinc finger transcription factor, is expressed in the mesonephros, the kidney, and the gonad [46]. In $Wt1^{-/-}$ mutants, the migration of PGC also occurs normally, but the mutation causes reduced size of gonads and increased cell death during gonadal formation in both sexes [47].

Lim homeobox protein 9 is expressed in the coelomic epithelium of the genital ridge. In $Lhx9^{-/-}$ mutants, the gonadal phenotype is very similar to that of $SF1^{-/-}$ and $Wt1^{-/-}$ mice [44]. They also show normal PGC migration into the genital ridge at E11.5, but no further development thereafter, and a complete loss of the gonad-like structures by E13.5 [43].

Steroidogenic factor 1 (Ad4BP; NR5A1), an orphan member of the nuclear receptor family of transcription factors, is expressed in the developing hypothalamus, the anterior pituitary, adrenal, and gonad [48]. $SF1^{-/-}$ mutants cause male to female sex reversal of both the internal and external genitalia, complete agenesis of adrenals and gonads, malformation of the hypothalamus, and abnormal function of the gonadotropes [48].

1.1.3: Selection of the ovarian differentiation pathway

The bipotential gonad is morphologically indistinguishable between males and females. However, sex-specific gonad differentiation begins when *Sry* (sex determining region of the Y chromosome) is expressed in the Sertoli cells in the male gonad between E10.5 and E12.0 [49, 50]. In the absence of *Sry*, the anti-testis activity of the ovary is first observed at E 11.5 - E12.5. Gene expression differences begin to be detectable between the ovary and the biopotential gonad [51]. A female sex determination gene has not been identified but there are at least three important candidate genes that include *Wnt4*, *R-spondin-1* (*Rspo-1*), and *Foxl2* [52-57].

Wnt4 has been shown in mice to regulate female sexual development through both negative and positive functions [58]. Expression of *Wnt4* is first observed in the mesonephric mesenchyme and coelomic epithelium at E9.5 [59]. *Wnt4* is required during the bipotential stage for initial Müllerian duct morphogenesis in both sexes and exhibits ovarian specific expression at the time of sex determination around E11.5 [52]. $Wnt4^{-/-}$ mutant females are masculinized, with an active Wolffian duct and no Müllerian duct and produce steroid enzymes [52, 60].

R-spondins are a protein family of growth factors that participate, via frizzled (FZD) receptors, in the regulation of development of some structures through stabilization of beta-catenin [61]. Thus, their ligand-type activities mimic those of the canonical Wnt ligands, resulting in transcriptional activation mediated by the beta-catenin/T cell factor (TCF) signaling pathway[62, 63]. In mice, *Rspo-1* is expressed in somatic cells with little in germ cells [64]. Deletion of murine *Rspo-1* causes partial sex reversal with ectopic steroid enzyme expressions and retention of the Woffian duct similar to the sex reversal in $Wnt4^{-/-}$ mutant females [57, 65]. *Rspo-1* is normally expressed in $Wnt4^{-/-}$ mutant ovaries while *Wnt4* expression is disrupted in $Rspo\text{-}1^{-/-}$ mutants, thus *Rspo-1* is upstream of *Wnt4* [61]. Expression of both *Rspo-1* and *Wnt4* in

the female gonad is essential for female sex determination for suppression of the male differentiation pathway and for maintaining ovarian function during female development through beta-catenin-mediated transcriptional activation [57, 61].

Foxl2 (Forkhead box L2) is a member of the large family of forkhead/winged helix transcription factors involved in ovarian development [53]. In mice, *Foxl2* is expressed in a female specific manner in the gonad from E12.5 and continues in pregranulosa cells through formation of early follicles [66, 67]. *Foxl2* plays a role in the autosomal female sex reversal phenotype of the polled intersex syndrome in goats. In *Foxl2* mutant mice, granulosa cells fail to pass from the squamous to cuboidal phase that precedes further proliferation of granulosa cells and oocyte growth [54, 67]. Thus, this gene is essential for granulosa cell differentiation and maintenance of ovaries. Also *activin-βa* and *MIS* (Müllerian inhibiting substance)/or *AMH* (Anti-Müllerian hormone), factors known as inhibitors of primordial follicle activation, are diminished or absent in Foxl2-/- mutant mice [67]. In addition, deletion of *Foxl2* revealed the ectopic expression of steroid enzymes such as steroidogenic acute regulatory protein (StAR), 17 alpha-hydroxylase (CYP17), and aromatase [68-70]. Thus, *Foxl2* expression in pregranulosa cells may be critical in granulosa cell-directed determination of other ovarian cell lineage fates [58].

1.1.3.1: Female germ cell specific meiosis

Sex-specific development of germ cells depends on their gonadal environment. At about E13.5, germ cells in female gonads enter meiotic prophase and pass through leptotene, zygotene and pachytene before arresting in diplotene around the time of birth [71]. In the male, however, meiosis is initiated after birth. Recently, the sexually dimorphic mechanism for meiosis has been explained by interactions between retinoic acid (RA) and CYP26B1 [72, 73]. RA stimulates

activation of Stra8 (Stimulated by retinoic acid gene 8), which induces entry into meiosis in both males and females. The developing ovary is exposed to high levels of RA, but the developing testis sees only low levels of RA because of the high quantity of the RA metabolism enzyme, CYP26B1[72, 73]. CYP26B1 was identified as a gene expressed in gonads of both sexes at E11.5, but is downregulated in the female and highly upregulated in the male gonads by E12.5. Loss of function of CYP26B1 allows entry of germ cells into meiosis in male gonads [73]. This observation suggests a mechanism by which the high level of RA and the low level of CYP26B1 allow expression of Stra8 and entry into meiosis in female gonads, whereas, the high level of CYP26B1 degrades RA and blocks entry into meiosis in male gonads.

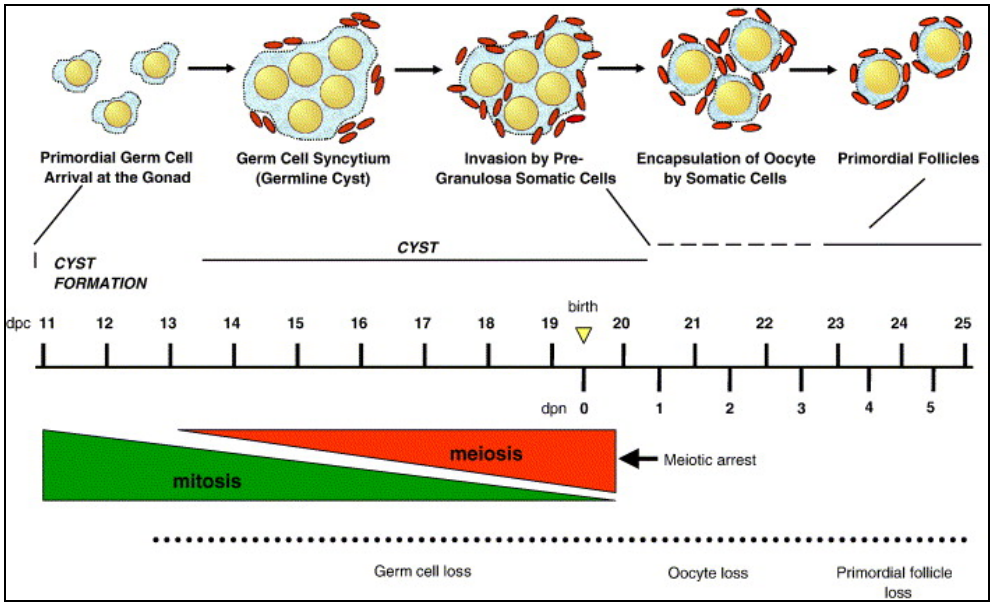
1.1.3.2: Communication between somatic and germ cells

The germ cell nest is an important developmental stage in the formation of the germline that is evolutionarily conserved in males and females of species ranging from higher insects to frogs, rodents and other vertebrates [74]. Female germ cells form germline cysts beginning at E10.5 (Fig. 1.3). These cysts were originally discovered in Drosophila and are essential for fertility [75, 76]. Germline cysts are held together by intercellular bridges [77] to form clonal clumps of up to 32 cells, that tend to go through mitosis synchronously [78]. The function of these cysts is unclear. Nests in oocyte development may help synchronize the germ cells for their entry into meiosis [78], and increase the store of materials and nutrients for later development [79].

To form individual follicles, somatic cells begin to invade between germ cells suggesting somatic cells mediate nest breakdown and primordial follicle development around birth [80]. The mechanism of nest breakdown is unclear, but several pathways have been implicated. Mice

lacking *Bmp15*, *Gdf9*, or *Foxl2*, or over-expressing *inhibin α* all exhibit an increase in multiple oocyte follicles (MOFs) after birth suggesting a defect in nest breakdown [81, 82]. Another theory is that neonatal exposure to diethylstilbestrol (DES), estradiol (E2), or the phytoestrogen genistein in mice induces formation of MOFs via the inhibition, or delay of nest breakdown [83]. Nest formation and breakdown are not well understood but are critical characteristic steps for normal follicle and ovarian development (Fig. 1.3).

Figure 1.3: Illustration depicting female germ cell development
(Adapted from Bristol-Gould. 2006)
Schematic of germline cyst formation, breakdown and primordial follicle formation. Following PGC arrival at the gonad, germline cysts form between 10.5 and 13.5 dpc. During cyst formation, mitotic division is accompanied by incomplete cytokinesis so that the daughter cells remain connected by intercellular bridges. Around 13.5 dpc, the first wave of oogonia within the cysts enter meiosis and arrest at the end of prophase I. Approximately 24 h after birth, these cysts begin to breakdown as somatic cells invade and begin to encapsulate individual oocytes into primordial follicles. Coincident with cyst breakdown is an initial loss of germ cells followed by primordial follicle loss.



1.1.3.3: Early folliculogenesis

The early stages of folliculogenesis are independent of gonadotropins and involve cell-autonomous and non-cell-autonomous factors [84]. The networks between somatic cells (granulosa cells) and germ cells (ooctyes) are necessary for successful follicle formation and initiation of follicle growth. In mice, nest breakdown is initiated at birth, and successful nest breakdown results in primordial follicles structured with oocytes surrounded by a single layer of flattened pregranulosa cells [85, 86]. Normal follicle development requires the presence of oocytes and, in the absence of oocytes, pre-granulosa cells transdifferentiate towards testicular tissue including XX Sertoli cells [87]. Earlier studies proposed that in the mouse ovary, the oocytes can grow faster when they are surrounded by about 10 cuboidal granulosa cells [88]. These observations imply that interactions between oocytes and pre-granulosa cells are critical for the initial formation of primordial follicles. The transition from primordial follicles to primary leads to an increase in size of the oocyte and conversion of the squamous granulosa cells into cuboidal. One striking characteristic of primary follicles is the appearance of the zona pellucida which surrounds the oocyte and is maintained throughout growth until the oocyte is ovulated.

Molecular control of early folliculogenesis requires specific genes in the germ cells such as Factor in the germline alpha (FIGLA), Newborn ovary homeobox gene (NOBOX) and Kit receptor (Kit), as well as in the surrounding somatic cells, or granulosa cells, such as Foxl2 and Kit-ligand (Kitl). These genes play critical functions during early folliculogenesis. Figla is a basic helix-loop-helix transcription factor [89]. In mice, Figla is expressed as early as E13.5 in the female gonad, and appears confined to oocytes of germ cell clusters and throughout folliculogenesis [90]. In Figla$^{-/-}$ mice, only female knockout mice are infertile, and the loss of

Figla does not affect germ cell migration or proliferation. However, after birth, primordial follicles do not form [91]. Nobox is a germ cell-specific transcription factor critical in early folliculogenesis and is expressed starting at E13.5 [92]. The deletion of the Nobox gene causes postnatal oocyte loss and failure of transition from primordial to primary follicles. In addition, the Nobox gene affects other important genes expressed in ooctyes such as Gdf9, Bmp15, Mos, and Oct4 [90]. Interaction between Kit and Kitl is critical for initiation of follicular growth and mutation of Kit or Kitl causes the failure of progression beyond the primary follicles [93, 94]. Disruption of receptor-ligand communications between somatic cells and the central germ cell may affect production of paracrine factors necessary for follicular growth [90]. Foxl2 also is an important gene for folliculogenesis. $Foxl2^{-/-}$ mice show failure of granulosa cell development, which is blocked at the squamous to the cuboidal transition. The deficiency of Foxl2 has no effect on the expression of oocyte-specific genes such as Figla and Kit, but the expression of Activin βA and Amh was reduced in the ovaries of $Foxl2^{-/-}$ mice [95-99].

1.1.4: Selection of the testis differentiation pathway

The bipotential gonad is morphologically indistinguishable between males and females. However, sex-specific gonadal differentiation begins when Sry (sex determining region of the Y chromosome) is expressed in the Sertoli cells in the male gonad between E10.5 and E12.0 in the mouse [49, 50]. Expression of Sry in the male gonadal primordium causes some of the somatic supporting cell precursors of the gonads to differentiate into Sertoli cells. The Sertoli cell is a key player that acts as an organizing center for testis development by directing all other cell types into their respective lineages [100]. Sry can alter chromatin structure and regulate transcription through its High Mobility Group (HMG) domain [101-103]. However, there is very little

information about the regulation of Sry. Recent studies lead to the genetic evidence that Sry induces up-regulated expression of SRY-related HMG box gene 9 (Sox9). Deletion or ectopic expression of either Sox9 or Sry induces the same effects: ovary development with deletion of either gene in XY gonads or testis development with ectopic expression of either gene in XX gonads [104-108]. In addition, Sox9 can functionally substitute for Sry and restore every aspect of male development, including male fertility [109, 110]. It is believed that Sox9 is not only necessary but also sufficient for male sexual development. Also, an interaction between SF1, Sry and Sox9 has been reported. SF1 and Sry cooperatively upregulate Sox9 and then, together with SF1, Sox9 also binds to the enhancer to help maintain its own expression after that of Sry has ceased [109]. Another factor, Fibroblast growth factor 9 (FGF9), is also known to be a regulator of Sertoli cell fate. Deletion of Fgf9 causes a failure of Sertoli cell differentiation and complete sex reversal [111-113]. Recent studies of interactions between Sry, Sox9 and Fgf9 reveal that Fgf9 is necessary to maintain Sox9 expression: without Fgf9, Sry gene retains normal expression, but Sox9 expression decreases following the downregulation of Sry [111, 113]. In addition, in in vitro culture, ectopic Fgf9 expression induced Sox9 and blocked Wnt4 in XX cells [113]. These data suggest a reciprocal manner of regulations between male and female gonads. First, Sry regulates the balance between Wnt4 and Fgf9 through the initial up-regulation of Sox9. Then Sox9 upregulates Fgf9 to maintain its own function [114]. This mechanism is very important for commitment to the testis pathway and preventing the ovarian pathway in XY gonads.

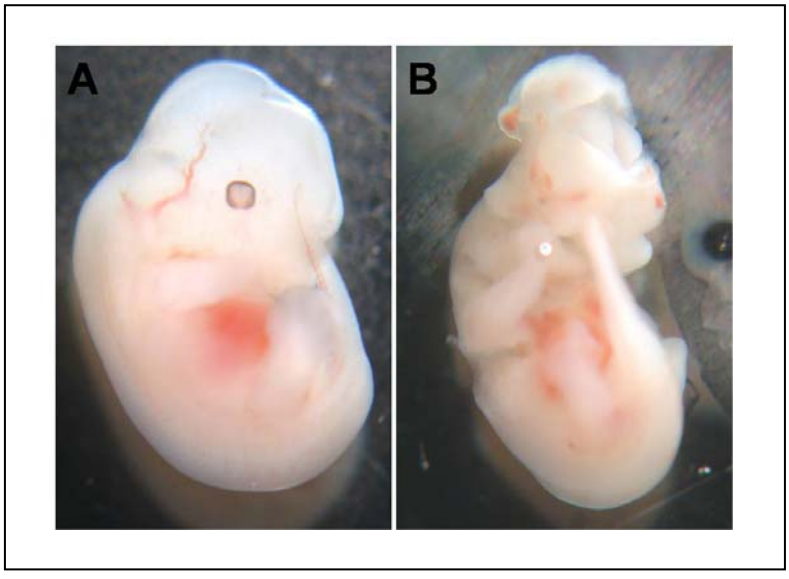
1.1.4.1: Early testis development

By E12.0 in the mouse, testis cords become visible in the male gonad, formed by Sertoli cells that surround clusters of primordial germ cells [115]. Sertoli cells in turn orchestrate the differentiation of other cell types in the embryonic testis: the steroidogenic Leydig cells within the interstitium, peritubular myoid (PM) cells enclosing the testis cords, and endothelial cells forming the vasculature. As a consequence of cell migration from the adjacent mesonephros into the male gonad and increased proliferation, the developing testis begins to enlarge noticeably. Interestingly, testis cords can still form in the genetic or pharmacologically induced absence of germ cells [116], demonstrating a negligible role of germ cells in this process. On the other hand, without the adjacent mesonephros, the testis will not form sex cords [117]. These findings suggest that in addition to the Sertoli cells, PM cells are also necessary [118]. Further evidence supports important interactions between neighboring somatic cells as well as between somatic and germ cells.

1.2: FUSED TOES (*Ft*) MUTANT MICE

The name of the Fused toes (*Ft*) autosomal dominant mutation is based on the phenotype of the heterozygous animals: a fusion of digits 1 to 4 of the forelimbs [119, 120]. The Fused toes (Ft) mutation is a 1.6-Mb deletion made by a transgene integration into region D of mouse chromosome 8 [121]. This deletion eliminated six genes: three members of the Iroquois gene family, *Irx3*, *Irx5* and *Irx6*, which form the *Irx B* cluster, and three other genes, Fused toes (*Fts*), Fantom (*Ftm*), and Fatso (*Fto* ,or fat mass and obesity associated) [122, 123]. Heterozygosity of the mutation leads to defects in programmed cell death such as fused toes on the fore limbs and thymic hyperplasia [120]. Homozygous *Ft* embryos die between E9.5 and E13.5 and present many abnormalities (Fig. 1.4). The limbs show anterior-posterior (AP) and dorsal-ventral (DV) polydactyly and distal truncations [124]. They also exhibit left-right asymmetry of heart looping and embryo turning is randomized [125]. The floor plate forms but is not maintained, and DV patterning of the spinal cord is perturbed [126].

Figure 1.4: Embryo morphology at E12.5.
A) Wild type embryo B) Ft mutant type embryo.



1.3: SIX MISSING GENES OF *Ft* MUTANT MICE

1.3.1: Iroquois homeobox genes

The *Iroquois* (*Irx*) genes were first discovered in *Drosophila*. Three *Irx* genes: *Araucan*, *caupolican*, and *mirror,* are clustered on one chromosome [127, 128]. In the mouse, these are grouped in two genomic clusters of three genes each [Figure 1-5]. Cluster *IrxA*, which contains *Irx1*, *Irx2*, and *Irx4*, is located at 25 cM of chromosome 13, and cluster *IrxB*, which contains *Irx3*, *Irx5*, and *Irx6*, is present at 43 cM of chromosome 8 in mice [129]. The same genomic organization is seen in humans, with cluster A and B located on chromosomes 5 and 16 respectively (Fig. 1.5). In the mouse, the expression patterns during development of *Irx1* and *Irx2* are almost identical in several tissues, and this is also true for *Irx3* and *Irx5* [130]. The expression of the third gene in each cluster, *Irx4* or *Irx6,* is in general more divergent. In some tissues, however, all the genes of a cluster, or even of both clusters, are identically expressed [131]. This suggests that some enhancers act on all the *Irx* genes of a cluster and that some of these enhancers are present in both clusters [130].

Iroquois proteins comprise a conserved family of homeodomain-containing transcription factors involved in patterning and regionalization of embryonic tissues in both vertebrates and invertebrates. *Iroquois* genes were discovered in Drosophila as neural prepattern genes regulating proneural genes in the achaete-scute complex (as-c) [132]. Loss of *Iroquois* genes alters neural differentiation, wing formation, and dorsoventral polarity in the head region[128, 133-136]. *Iroquois* genes have been isolated from several vertebrates. Xenopus *iro1*, *iro2*, and *iro3* induce proneural markers including the as-c homolog Xash3 [137, 138]. In the chicken, *Irx4* is expressed in heart ventricles and regulates ventricle/atrium cell fate determination [139]. Zebrafish *iro3* was reported to be expressed in the notochord in the late gastrula and in the neural

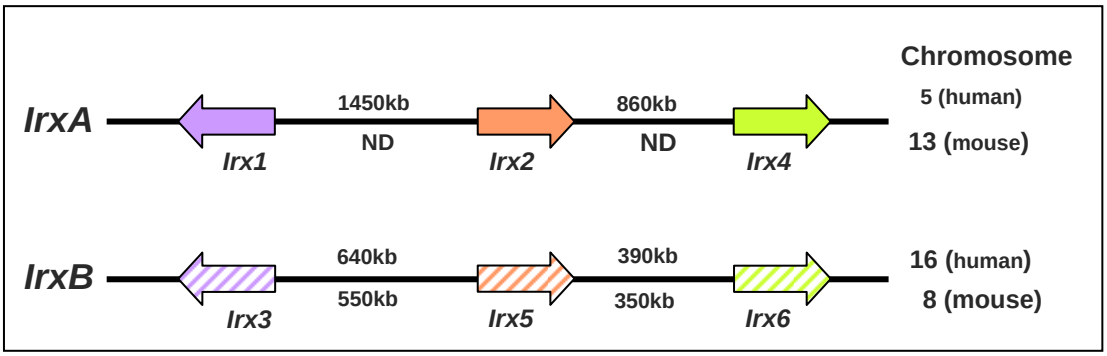
tube during somitogenesis [140]. In addition to these early roles in embryonic development, *Irx3* and its orthologues have a later role in patterning of the vertebrate spinal cord and the forebrain. *Irx3* is crucial for the correct specification of interneurons that arise from the intermediate spinal cord [141] and it also regulates differential competence of thalamic and prethalamic primordium in the forebrain in chickens [142-144]. In addition to neuronal specification, *Irx3*, along with *Irx1*, *2*, *4*, and *5*, has been implicated in patterning of the embryonic heart in the mouse [145].

Whereas *Irx3* is a central figure in axis formation early in development and in determining cell fate later on, it stands to reason that it is the mediator of inductive signals from very diverse signaling families. For example, *Irx3* defines regionally distinct competence by mediating Shh and TGF-β signaling molecules in the developing forebrain. It acts by repressing transcription of other Hox genes to demarcate a clear boundary for the zona limitans intrathalamica and ventral spinal cord [144]. Several other signaling factors have been implicated in regulating *Irx3* besides Shh [141, 143, 144, 146] and include FGF8 [147], EGF [148, 149], Wnts [143], Nodal factors[150], and BMPs [151].

In mouse gonads, *Irx3* is expressed at a much higher level in female versus male gonads during gonadal development and is restricted to the somatic cell component of XX gonads [1]. In a recent study, Wnt4 signaling was shown to induce *Irx3* gene expression to maintain female germ line cysts [152]. These data suggest *Irx3* is an important factor for normal female gonadal development. In addition, *Irx5*, one of the *IrxB* cluster genes, shows similar expression patterns to *Irx3*. Homozygous Irx5$^{-/-}$ mice are viable and fertile but have defects in differentiation of retinal cone bipolar cells. They also are slightly smaller than their wild-type counterparts [153]. Homozygous *Irx3* or *Irx5* knockout mice are viable and fertile but knockout of both *Irx3* and *5*

results in early embryonic death around E13.5. These findings suggest that *Irx3* and *5* likely compensate for the loss of the other gene.

Figure 1.5: Schematic representation of the genomic organization of mammalian *Irx* gene clusters on mouse chromosomes 13 and 8 and human chromosomes 5 and 16.
Adapted from *Arjan C. Houweling et. al. 2001 Mech. Dev*



1.3.2: The other three genes of the *Ft* mutation; *Ftm*, *Fto*, and *Fts*

The *Ftm*, *Fto*, and *Fts* genes are closely linked on mouse Chromosome 8 [154]. The *Ftm* (fantom; *Rpgrip1l*- Mouse Genome Informatics) gene was originally identified in the mouse fused toes mutation *Ft* (fused toes; also known as *Fts* – Mouse Genome Informatics). Recent studies found that the *Ftm* gene encodes a protein that is localized to the basal body of cilia and is essential for GLI protein function to mediate Hh signals. $Ftm^{-/-}$ mice die shortly after birth with defects reminiscent of the *Ft* mutant mouse model [155]. The *Fto* (fat mass and obesity associated) gene resides next to *Ftm* but is transcribed in the opposite direction [156]. Recently, the *Fto* gene was found to encode a protein that can regulate obesity and diabetes [123, 157-162] through energy intake and metabolism regulation [163]. $Fto^{-/-}$ mice show increased postnatal death and growth retardation [164]. Finally, *Fts* (*Ft1*) is highly expressed in brain, kidney, and

testes and is detectable in all other adult organs and throughout embryonic development. The *Fts*$^{-/-}$ mouse has no discernable phenotype [165].

Here I report that all three genes, *Ftm*, *Fto*, and *Fts*, are present in both male and female gonads during embryonic and adult stages. In particular, the *Ftm* and *Fts* genes are expressed at higher levels in female gonads than male gonads during embryonic gonadal development.

1.4: RESEARCH OBJECTIVES

The process of formation of either an ovary or testis from a bipotential gonad is a very special event in mammalian development. Many molecules and signaling pathways are required to achieve normal development of either ovaries or testes. Homeobox genes are well known to play an important role for gonadal development as well as embryo morphogenesis and organogenesis. Previously, our published results suggested that *Irx3* might be important for early female gonadal development, but its function is still unclear. I will discuss more about the functions of the *IrxB* gene cluster along with three other genes, *Fts*, *Ftm*, and *Fto*, in the context of the *Ft* mutant mouse and the consequences of the loss of these genes in ovary and testis formation.

The first specific aim of my doctoral studies was to determine the function of the *Ft* locus during ovary development. In Chapter 2, I report that the *Ft* locus is critical for normal ovarian development from early gonadal development to follicle formation. The genes within the *Ft* locus are important for germ cell proliferation and somatic cell organization during early gonad development. Additionally, during folliculogenesis, these genes are essential for maturation of follicles from primary to secondary follicles.

The second specific aim was to investigate the function of the *Ft* locus during testis development. In Chapter 2, I show that the *Ft* locus genes were necessary for female ovary development. In Chapter 3, I report that the *Ft* locus is not required for prenatal testis development, but contributes to normal testicular structure (Sertoli cell architecture) after birth.

The third and final specific aim was to demonstrate the function of the *IrxB* gene cluster, especially *Irx3* and *5*, during ovary and testis development. In Chapter 4, I provide preliminary

results that suggest that these two genes are not necessary for early germ cell migration and proliferation in male or female gonads, but are essential for ovary maturation after birth.

My dissertation first proposes that the *Ft* locus genes are newly discovered genes that are necessary for normal ovary and testis development. First, *Ftm*, *Fto*, and *Fts* genes are required for normal germ cell development during early gonadal formation including germ cell migration from the hindgut and germ cell proliferation in gonads from both males and females. There are no studies about the functions of these genes during primordial germ cell migration and proliferation. Second, the *Ft* locus genes are required for normal follicle development. Deletion of this locus induces little germ cell production, and failure of normal follicle formation (most mutant follicles are blocked at primary follicles). Third, the *Ft* locus genes are required for postnatal testis development through regulation of Sertoli cell development. My results suggest that deletion of this locus induces failure of Sertoli cell maturation. Therefore, abnormal Sertoli cells lose their functions to support germ cells. Last, *IrxB* cluster genes, especially *Irx3* and *Irx5*, are more involved in female follicle development. The loss of two genes, *Irx3* and *Irx5*, induces a decrease of normal follicles and a partial disruption of granulosa cell organization. In males, however, I have evidence that these two genes do not function in testis development. The conditional double-knockout mouse which lacks *Irx3* and *Irx5* shows normal testis development. This thesis provides the first glimpse into the functioning of the *Ft* locus genes: *Irx3*, *Irx5*, *Irx6*, *Ftm*, *Fto*, and *Fts* during ovary and testis development.

CHAPTER 2

FAILURE OF GERM CELL PROLIFERATION AND DEGENERATION OF FOLLICLES DURING OVARIAN DEVELOPMENT IN FUSED TOES MUTANT MICE

2.1: ABSTRACT

Active communication between germ cells and their supporting cell lineages is absolutely essential for normal ovarian development. When germ cells are lost, neighboring somatic cells become totally dysfunctional or transdifferentiate to a new developmental fate. Very little is known about the consequences of abnormal somatic cells may incur on germ cell maturation. Here I demonstrate that disruption of a cluster of six genes on murine chromosome 8, as exemplified by the Fused Toes (*Ft*) mutant mouse model, results in formation of ovaries that eventually contain few follicles. The six genes within the *Ft* locus include the *IrxB* cluster (*Irx3, -5, -6*), *Fts*, *Ftm*, and *Fto*, of which *Irx3*, *Irx5*, *Ftm* and *Fts* are expressed at greater levels in developing ovaries versus testes. All six genes were detected in somatic cells of the developing ovary; only *Ftm* was also expressed in germ cells. I report two fundamental defects that are likely caused by disruption of different combinations of genes within the *Ft* locus. First, primordial germ cells migrate through the hindgut normally, but many fail to colonize the nascent ovary, those that do migrate into the ovary fail to proliferate after arrival. Second, developing granulosa cells form follicles with the few remaining germ cells, but these cells degenerate over time culminating in abnormal follicles that fail to mature past the primary stage. Ultimately, the demise of the developing *Ft* mutant ovary is caused by deficits in both germ cells and somatic cells as a result of disrupted expression of genes that are localized primarily to somatic cells. Thus, these data suggest that genes within the *Ft* locus contribute information from

somatic cells to affect development and maturation of both germ and somatic cell lineages to form a functional ovary.

2.2: INTRODUCTION

The fate of ovarian structure development depends on communication between somatic supporting cells and primordial germ cells (PGC) that mature together. During fetal and perinatal development, somatic cells require the presence of germ cells to maintain their identity in XX gonads. Genital ridges devoid of oogonia will initiate formation of pre-follicular cells, but differentiation soon ceases and the developing gonad will eventually degenerate to a streak gonad containing only stromal tissue [166]. If germ cells are lost after the onset of meiosis, female supporting cells will transdifferentiate into Sertoli cells and promote ectopic expression of characteristically male genes [167-169]. Once follicles have matured beyond the primary stage, however, ablation of oocytes has no effect on granulosa cell identity [169, 170]. Ultimately, if there are too few primordial follicles capable of transition to the primary stage by the early postnatal period, the ovary cannot recover and consequently suffers long-term subfertility or premature failure. Some factors associated with this fate include FIG1α, NOBOX, and BMP15 [171-173].

Somatic cells also express their own factors to communicate supportive signals to oocytes in addition to promoting ovarian infrastructure. Recent discoveries have implicated Wnt signaling via β-catenin (Ctnnb1) and R-spondin (Rspo1) in maintaining the female somatic cell phenotype [174-178]. Independent of the Wnt pathway, the transcription factor, FOXL2, has also been shown to be essential for normal differentiation and maintenance of the female supporting lineage. Ablation of the *Foxl2* gene in mice causes defects in granulosa cell development late in

development. In addition, conditional disruption of the gene in the adult ovary of mice induces transdifferentiation of the ovary into a functional testis [170, 179]. Furthermore, it has been established in other species that *Foxl2* mutations cause sex reversal [180, 181]. Thus, at a minimum, a combination of Wnt-related factors and *Foxl2* work independently to sustain ovarian development; however, the molecular and cellular mechanisms by which these events occur are unclear.

One target of Wnt signaling that exhibits ovary-specific expression during development is IRX3 [176, 182-184]. *Irx3* is a member of the Iroquois homeobox transcription factor family that includes six family members clustered in two groups of three within the *IrxA* cluster (*Irx1*, -*2*, -*4*) on chromosomes 13 and the *IrxB* cluster (*Irx3*, -*5*, -*6*) on chromosome 8 in the mouse [129]. In general, clustered family members share similar expression patterns and only the *IrxB* gene cluster, especially *Irx3* and -*5*, is expressed in mouse gonads [130, 185]. Previously, we reported that *Irx3* expression is limited to somatic cells within female gonads during prenatal development [183]. In agreement with other studies, we report herein that *Irx5* shares the same expression profile as *Irx3* within developing ovaries. Based on these findings, we hypothesized that the female-specific pattern of the *IrxB* gene cluster would be important for ovarian development. Currently, there are no reports of functional studies for *Irx3* in mice. However, $Irx3^{-/-}$ or $Irx5^{-/-}$ mice are fertile suggesting that these factors likely compensate for each other and implies that optimal assessment of the function of these genes requires elimination of the entire *IrxB* cluster (C.C. Hui, University of Toronto, personal communication and reported herein).

The *Ft* mutation is a 1.6-Mb deletion on mouse chromosome 8 that eliminates six genes including the *IrxB* cluster and three other genes, *Ftm, Fto and Fts* [129]. The fused toes name describes the phenotype of heterozygote animals that have fusion of digits 1 to 4 in the forelimbs

[119, 120]. These animals also develop hyperplasia of the thymus and both defects have been attributed to defects in programmed cells death [120, 186]. In the homozygous state, *Ft* mutant mice suffer severe abnormalities in brain, spinal cord, craniofacial, heart, and limb development and embryos die between E10.5 and E14.5 [119, 120, 124-126, 187]. The *Ft* mutant mouse has been used as a model to investigate the function of the *IrxB* cluster genes because of the functional redundancy of *Irx* genes and the strong correlation between their expression profile and locations of defects within these mice [126, 187].

We report here that four of the six missing genes in *Ft* mutant mice, *Irx3, Irx5, Ftm* and *Fts*, exhibit expression within somatic cells of fetal gonads with highest levels in the developing ovary. Ovaries from *Ft* mutant mice degenerate over time beginning with reduced germ cell numbers caused by defective migration into the ovary and failure of proliferation during early gonadal development. As the ovary develops, female somatic cells fail to mature alongside the few remaining germ cells ultimately leading to disturbed follicle structures and arrested progression of follicles at the primary stage. These developmental mishaps caused by genes within the *Ft* locus may provide additional insight into unexplained causes of premature ovarian failure.

2.3: MATERIALS AND METHODS

2.3.1: Animals

Heterozygote female and male *Ft* mutant mice were paired together and checked for the presence of a vaginal plug the next morning. The day when the vaginal plug was detected was considered 0.5 day of gestation, or E0.5. Gonads were collected at E11.5 or E12.5 because homozygous *Ft* mutant embryos died between E10.5 and E14.5 depending on background strain. Our colony was maintained in a C3H background. The gonads were cultured in DMEM with 10% FBS or were transplanted under the kidney capsule of a nude mouse host. For genotyping, embryo tail bits were digested in 50 mM sodium hydroxide and subjected to standard PCR using gene specific primers: Ft:5'-GTCCTTTCTCCATGGGTATG-3', Wt: 5'-GTGGAACCCTTCTGTACATG- 3', Ft/Wt: 5'-CTGAAAGGTTGTACTGAGCC-3', which allowed discrimination between the three genotypes. For sex genotyping, primers 5'-TGCAGCTCTACTCCAGTCTTG-3' and 5'-GATCTTGATTTTTAGTGTTC-3' were used to detect the presences of the *Sry* gene. All procedures described were reviewed and approved by the Institutional Animal Care and Use Committee at University of Illinois and were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals. All experiments were performed on at least five animals for each genotype.

2.3.2: RNA extraction and real-time PCR

RNA was prepared from pooled individual gonads (separated from mesonephros) from E12.0-E15.5 gonads, testes, and ovaries using the RNeasy Micro Kit (Qiagen). Adult ovaries and testes were collected from 6-7 week-old mice. All real-time PCR assays were carried out using the SYBR Green I Kit (Applied Biosystems) as previously described [183]. The relative

expression level for each sample was determined in the same run and was expressed as the ratio of the quantity of RNA of interest to that of a control RNA (36B4). Gene specific primers were designed using Primer Express; primer sequences are shown below (Table 2.1).

2.3.3: Germ cell depletion by busulfan treatment

Pregnant female mice were administered an intraperitoneal injection of 100 μℓ Busulfan solution (16 mg/ml in 50% DMSO) or 50% DMSO (control) at E9.5 as previously reported [183]. Ovaries were harvested from embryos from the treated females at E13.5 for RNA isolation and quantitative real-time PCR.

2.3.4: Ovary transplantation

Ovaries were harvested at E12.5 from wild type or *Ft* mutant embryos and then transplanted underneath the kidney capsule of an ovariectomized nude mouse of at least 8 weeks of age (Charles River Laboratory). Ovariectomy was performed 10-14 days before transplantation surgery. For kidney capsule transplantation, a dorsolateral incision was made into the skin and peritoneum to expose the kidney. A small incision was made into the kidney capsule and a pair of wild type or *Ft* ovaries was inserted under the capsule. The kidney was then returned to its normal anatomical position. The body wall was sutured, and the skin was closed with wound clips. Grafted ovaries were recovered 1, 2, and 3 weeks after transplantation.

2.3.5: Immunohistochemistry

Ovaries and transplanted tissues were fixed in 4% paraformaldehyde in PBS at 4℃ overnight and then rinsed three times in PBS for 5 min each. Samples were then put through a sucrose gradient (10%, 15% and 20%) followed by incubation in 1:1 20% Sucrose and OCT-

freezing media (Tissue-Tek) at 4℃ overnight. The samples were embedded in a 1:3 20% sucrose and OCT cocktail and cut into 10μm frozen sections. Primary antibodies were as follows: goat anti-MIS (1:250; Santa Cruz Biotechnology), goat anti-GATA4 (1:250; Santa Cruz Biotechnology), rabbit anti-VASA (1:100; Santa Cruz Biotechnology), mouse anti-Tra98 (1:250; Cosmo Bio), rat anti-mouse CD1 (1:100; BD Biosciences), rabbit anti-γH2A.X (1:250; Millipore), rabbit anti Phospho-Histone H3 (1:250; Cell signaling), goat anti-FOXL2 (1:250; Everest Biotech), and rabbit anti-LAMININ (1:250; Sigma-Aldrich). All secondary antibodies (FITC or Rhodamine-conjugated donkey anti-rabbit, goat, and mouse antibodies) were obtained from Jackson Laboratory and used at a dilution of 1:300. Images were collected on a Nikon E600 microscope and processed using Adobe Photoshop.

2.3.6: Histology

Samples were fixed with 4% paraformaldehyde (PFA) in PBS overnight at 4℃, processed through graded ethanol series, embedded in paraffin, sectioned to 5μm, deparaffinized, rehydrated and stained with hematoxylin and eosin. Slides were mounted using Permount. Plastic sections were used to study the ultrastructure of transplanted ovary tissues. Ovaries were fixed in 4% glutaraldehyde overnight and kept in 2% glutaraldehyde at room temperature or 4℃. Tissue processing and staining (Toluidine blue/basic Fuchsin staining) were performed in The Center for Microscopic Imaging, College of Veterinary Medicine, University of Illinois. The procedures are based on previously described methods [188].

2.3.7: Oct4 whole-mount embryo immunohistochemistry

E9.5 embryos were fixed in 4% paraformaldehyde (PFA) and then washed in PBS. Embryos were blocked in PBSST (PBS/0.5% Trition X-100 with 5% donkey sera, 2x1 hour

washes) and incubated overnight at 4℃ in PBSST with goat anti-Oct-3/4 (Santa Cruz Biotechnology; 1:100 dilution) antibody. The following day, the embryos were washed in PBSST (3X 1 hour) at room temperature and then incubated overnight at 4℃ in PBSST with doney anti-goat-FITC-conjugated secondary antibody (Jackson Laboratory; 1:300 dilution). The embryos were washed in PBSST (5X 1 hour) and then mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA).

2.3.8: Germ cell counts

Total numbers of PGC in E9.5 hindguts were counted using a fluorescence microscope after staining for Oct3/4. Germ cells of E12.5 gonads and E12.5 gonads cultured for 2 days cultured gonads were counted using at least 5 different biological samples for each genotype using a previously described method [189]. Briefly, gonads were stained using antibodies against PECAM-1 or TRA98 and imaged using a fluorescence microscope. Longitudinal sections from the central third of each ovary were selected. For each ovary, all PECAM-1 or TRA98-positive cells were counted. To quantify the percentage of mitotic germ cells at each stage, the number of germ cells in each section positive for both phosphorylated histone H3 and PECAM-1 labeling or both phosphorylated histone H3 and TRA98 labeling were counted and divided by the total number of PECAM-1 or TRA98 positive germ cells in the section.

2.3.9: Statistical analysis

Statistical differences were determined using two-tailed Student's t test comparisons.

Table 2.1: PCR primers for real-time PCR (written 5' to 3')

Gene	Forward primer	Reverse primer
Irx3	CGCCTCAAGAAGGAGAACAAGA	CGCTCGCTCCCATAAGCAT
Irx5	GGCTACAACTCGCACCTCCA	CCAAGGAACCTGCCATACCG
Irx6	AGCACATCCCAGTTTCTGGTGTCT	ACAGCAAAGAGTAGAGGCAGAGGT
Fts	TTCACCCACTAGTTGATCCCACCT	ATGGTTATGGTTCCGCCTCCACTT
Ftm	TGCAGCGTGTCAGTTGAGATTCCA	TATCCTAATGCCTTTGCCCGCTCA
Fto	TGCGAAGGCTCTGAGGATGAAAGT	AATCCTGGTGTCTCGATGTCCCAA
Vasa	GAGATTGCCTTCAGTACCTATGTG	GTGCTTGCCCTGGTAATTCT
Foxl2	GCAAGGGAGGCGGGACAACAC	GAACGGGAACTTGGCTATGATGT
SDF-1	GAGAGCCACATCGCCAGAG	TTTCGGGTCAATGCACACTTG
Bmp-2	AAGCGTCAAGCCAAACACAAA	GAGTTCAGGTGGTCAGCAAGG
Bmp-4	TTCCTGGTAACCGAATGCTGA	CCTGAATCTCGGCGACTTTTT
Bmp-5	AGCCTGCAAGAAGCACGAAC	AAAAGAACATTCCCCGTCACAA
Bmp-6	CAACGCCCTGTCCAATGAC	ACTCTTGCGGTTCAAGGAGTG
Bmp-7	GATTTCAGCCTGGACAACGAG	GGGCAACCCTAAGATGGACAG
36B4	CGACCTGGAAGTCCAACTAC	ATCTGCTGCATCTGCTTG

2.4: RESULTS

2.4.1: Genes located within the *Ft* mutation have a higher expression in female gonads

Previously, we reported that *Irx3* expression increases in developing ovaries from E12 to E13.5, remains elevated until birth, and then rapidly declines shortly after birth. At the same time, *Irx3* transcripts remain very low throughout development in the male gonad [183]. We isolated RNA from gonads of wild type male and female mice at several developmental stages to assess transcript levels of the genes present within the *Ft* mutation besides *Irx3*; these include *Irx5, Irx6, Ftm, Fto*, and *Fts*. All six genes were expressed in both male and female gonads with four genes, *Irx3, Irx5, Ftm*, and *Fts* demonstrating greater transcript levels in female versus male gonads during development. The expression pattern for *Irx5* followed the same pattern as *Irx3* with the exception of the appearance of significant transcript levels in adult testis samples (Fig. 2.1A-B). Transcript measurements of *Ftm* and *Fts* were similar to each other in demonstrating equivalent expression in male and female gonads at E12 and E13.5 followed by increased expression in female gonads from E15.5 to P4 (Fig. 2.1D-F). In both cases, ovarian expression decreased in adult tissues; *Ftm* transcripts were dramatically increased in the adult testis (Fig. 2.1D). In general, expression profiles of *Irx6* and *Fto* were similar between male and female gonad samples (Fig. 2.1C,E).

Given the predominantly female oriented expression profile of the genes located within the *Ft* mutation, we focused the remainder of this study on development of the female gonad. IRX3 has been localized to somatic cells and its expression does not depend on the presence of germ cells in female gonads [190]. Analyzing EGFP (Enhanced green fluorescent protein) activity in gonads from mice containing the EGFP reporter gene within the *Irx5* locus ($Irx5^{EGFP/+}$) at E13.5, we found that like IRX3, IRX5 localized to somatic cells in female gonads

(Fig. 2.2A-B). Both male and female Irx5$^{EGFP/EGFP}$ mice are fertile, which confirmed previous communications of the fertility status of *Irx5*-disrupted mice (Personal communication with C.C. Hui, unpublished data). To gain information regarding which cell types express each gene within the *Ft* mutation, we measured transcript levels using XX gonads harvested from E13.5 embryos exposed to busulfan, an agent known to deplete maternal and embryonic gonads of germ cells [191]. Our results showed complete loss of v*asa* transcripts verifying the efficacy of busulfan treatment. As expected, no difference in *Irx3* or *Irx5* expression was detected between control and busulfan treated samples (Fig. 2.3). Results also show that busulfan treatment had no effect on expression of *Irx6, Fto,* or *Fts* suggesting that each of these genes are expressed primarily in somatic cells at E13.5 (Fig. 2.3). A significant decrease in transcripts was detected in *Ftm* after busulfan treatment; however, RNA levels were not completely depleted suggesting the gene is expressed in both somatic and germ cells.

2.4.2: *Ft* mutant ovaries contain fewer germ cells

Ft mutant embryos die between E10.5-14.5 depending on the mouse strain [120]. In our colony, *Ft* mutant embryos are maintained on a C3H background and die shortly after E12.5. Female gonads from *Ft* mutant embryos were present at E12.5 and were morphologically indistinguishable from wild type and heterozygote littermates. However, XX gonads stained with PECAM-1 (platelet/endothelial cell adhesion molecule 1, expressed in both germ cells and endothelial cells and are distinguished by shape) demonstrated a substantial decrease in germ cell numbers when *Ft* versus wild type embryos were compared at E12.5 (Fig. 2.4A-B). Similar results were obtained when analyzing E12.5 XX gonads subjected to two days explant culture and stained with another germ cell-specific marker, Tra98 (Fig. 2.5A-B). To quantify these

observations, germ cells were counted on sections of mutant and control ovaries stained with PECAM-1. Germ cell numbers were statistically decreased in XX gonads from *Ft* mutant mice at both time points (E12.5: wild type 188 ± 20, n = 14; ft/ft 73 ± 14, n = 8; E12.5 plus two-day culture wild type 133 ± 18, n = 10; ft/ft 59 ± 11, n = 8) (Fig. 2.4C; Fig. 2.5C). Quantitative real-time PCR measurements of *Vasa* transcripts verified a decrease in germ cell presence in *Ft* mutant gonads from female embryos at E11.5, E12.5, and E12.5 plus two days culture (Fig. 2.6).

Germ cells enter the developing gonad at the anterior end and eventually occupy the entire structure as they proliferate [192]. During this time in the female gonad, dividing germ cells remain interconnected and are organized into nests as they are surrounded by a basement membrane formed by a layer of somatic cells [78]. This architecture is illustrated in XX gonads from wild type embryos at E12.5 and E12.5 plus two-day culture, but is not appreciated in the *Ft* mutant gonad (Fig. 2.4B; Fig. 2.5B). Instead, most germ cells in XX gonads of *Ft* mutant embryos are present in isolation (Fig. 2.4B; Fig. 2.5B) and neighboring somatic cells appear bunched together and disorganized (Fig. 2.5B, arrows). When germ cell cysts did appear within the mutant ovary, they were predominantly located at the anterior end. In addition, germ cells were completely absent in the posterior half of the XX gonad in over 40% of mutant ovary samples (Fig. 2.4B'). Together, these data indicate a severe defect in germ cell numbers and abnormal placement of remaining germ cells in the ovaries of *Ft* mutant embryos.

The clumps of somatic cells expressed the female somatic cell marker, FOXL2, and clumping was likely the result of fewer germ cells (Fig. 2.5B, arrows). However, because it has been reported that somatic cells that have lost their corresponding oocytes may transdifferentiate into Sertoli cells during early ovarian development [167-169], we investigated whether somatic cell clumps expressed Sox9 at two different time points, E12.5 and E18.5 (one week after

transplantation). No Sox9 was detected at either time point suggesting the granulosa cell phenotype was maintained (Fig. 2.7).

To determine whether the lack of germ cell numbers was a consequence of events that occurred before or after colonization of the developing gonad, migrating primordial germ cells were visualized with an OCT3/4 antibody within the hindgut of embryos collected at E9.5. There was no difference in PGC numbers detected between wild type and *Ft* mutant embryos during migration (wild type 210 +/- 24, n=4; *Ft* mutant 191.5 +/- 18 n=4; $p<0.36$) (Fig. 2.8A-C). In addition, transcript levels of the PGC homing signal to the gonad, *sdf1* [193], were not different when wild type and *Ft* mutant ovary samples were compared at E11.5 and E12.5 (Fig. 2.9). Together, these data suggest that germ cells proliferate normally during migration.

2.4.3: Reduction in germ cell proliferation in *Ft* mutant gonads

Primordial germ cells proliferate as they migrate into the gonad. By E14.0, mitosis ceases in germ cells of XX and XY gonads and meiosis ensues in the female gonad [194]. Female gonads at E12.5 and E12.5 plus two days culture were assessed to determine whether the decrease in germ cell numbers within *Ft* mutant gonads was caused by a defect in proliferation or increased cell death. No difference in apoptosis was detected using activated caspase-3 or TUNEL staining between wild type and *Ft* mutant gonads at either stage (data not shown).

Results from proliferation studies showed that germ cells co-stained with antibodies against phosphorylated histone H3 (p-HH3), a mitosis marker, and PECAM-1 or TRA98 were easily identified within wild type gonads (Fig. 2.10A; 2.11A; white arrows). In contrast, doubly stained germ cells were rare within *Ft* gonads (Fig. 2.10B; 2.11B). The average percentage of germ cells positive for p-HH3 staining was significantly lower in *Ft* mutant gonads at both stages

(E12.5: wild type 6.3 ± 0.3%, n = 8; ft/ft 2.1 ± 0.7%, n = 8; 2days cultured gonad: wild type 7.2 ± 1.3%, n = 8; ft/ft 2.6 ± 0.7%, n = 8, *$p < 0.01$) (Fig. 2.10C; 2.11C).

Although several factors have been associated with PGC proliferation during migration, signals for stimulating PGC proliferation once they arrive within the gonad are unknown. Potential factors include the bone morphogenic protein (Bmp) family members and Bmp7 has been associated with PGC proliferation within the early testis [195, 196]. Using quantitative real-time PCR, we detected no difference in transcripts between wild type and *Ft* mutant gonad samples for Bmp family members: *Bmp2, -4, -5, -6,* and *-7* (Fig. 2.12). These data suggest that the *Ft* mutation includes genes that are critical for maintaining PGC proliferation within the developing gonad; further studies are required to identify the mechanism behind this defect.

2.4.4: Normal follicles fail to form in *Ft* mutant ovaries

Studies from early stage *Ft* mutant ovaries suggest that development is abnormal and prompted additional studies to assess PGC and ovary maturation. Because homozygote *Ft* mutant embryos die around E12.5, gonads were harvested at E11.5 from wild type and *Ft* mutant embryos and transplanted them under the kidney capsule of a nude mouse host to evaluate ovarian development over time. Histological examination of transplanted ovaries from wild type female embryos demonstrated normal progression of germ cell nests into single follicles that could mature into pre-antral follicles by three weeks after transplantation (Fig. 2.13A,C,E). In contrast, germ cell nests persisted alongside single follicles in *Ft* mutant ovaries (Fig. 2.13B,D,F). In addition, the follicles within the *Ft* mutant ovary contained germ cells that were substantially smaller than wild type and were surrounded by a single cell layer of somatic cells

that were either fusiform (Fig. 2.13D, black arrows) or cuboidal (Fig. 2.13F, red dotted line) in shape.

Germ cell maturation was monitored in transplanted ovaries over time. Samples taken seven days after transplantation, which is approximately equivalent to P0 or birth, were evaluated for progression into meiotic division. Although there were very few germ cells identified in *Ft* mutant ovaries, there was evidence that they could enter into meiotic division (Fig. 2.14A-B). Examination of follicles after two and three weeks transplantation (equivalent to approximately P7 and P14, respectively) showed that follicles in wild type ovaries progressed from primary to pre-antral stages and germ cells increased in diameter (Fig. 2.14C,E). In contrast, germ cell-containing follicles within *Ft* mutant ovaries were increasingly difficult to identify over time and germ cells failed to grow (Fig. 2.14D,F,G).

Follicle morphology and integrity of granulosa cells were also assessed in transplanted ovaries over time. As noted above, wild type ovaries contained progressively more mature follicles from two to three week transplantation samples (Fig. 2.13C,E; Fig. 2.14C,E). In addition, expression of granulosa cell markers, Müllerian inhibitory s,ubstance (MIS) and FOXL2, clearly demarcated individual follicles that were surrounded by a normal basement membrane illustrated with laminin staining (Fig. 2.14H,J). Granulosa cells within *Ft* mutant ovaries expressed both MIS and FOXL2, but in irregular patterns and at substantially lower levels than wild type cells (Fig. 2.14I). Transcripts of *Foxl2* were significantly lower earlier in ovarian development even though protein detection did not appear affected (Fig. 2.15) (Fig. 2.5A,B). The protein expression patterns within mutant ovaries identified some granulosa cells that were organized as follicles, but also recognized disorganized clumps of cells that lacked germ cells (Fig. 2.14I). These clumps of granulosa cells did not express SOX9 suggesting that

there was no transdifferentiation to Sertoli cells in the absence of germ cells (Fig. 2.7). Adding to the apparent disarray of ovarian structure, laminin staining demonstrated a significant disruption of the basement membrane surrounding follicles and granulosa cells within mutant ovaries (Fig. 2.14K). To summarize these data, we conclude that there is a severe communication failure between disorganized and degenerating granulosa cells and neighboring germ cells that stunt follicle maturation in *Ft* mutant ovaries.

2.5: DISCUSSION

Although much is known about how PGC affect somatic cell fate, little is known about how somatic cells promote the proliferation and differentiation of PGC during early ovarian development. Several events occur within *Ft* mutant ovaries to culminate in failure of follicle maintenance and maturation. First, few PGC successfully inhabit the gonad, and upon arrival, they fail to proliferate and in many cases they were unable to inhabit the entire gonad with individual germ cells and germ cell nests were predominantly confined to the anterior ovary. Second, follicles that did manage to form were unsuccessful in progressing beyond primary follicles. Finally, as follicles within *Ft* mutant ovaries progressed over time, their germ cells did not grow and their neighboring granulosa cells began to degenerate as evidenced by decreased expression of MIS and FOXL2 and deterioration of the basal lamina. By approximately P14 (after three weeks transplantation), follicles were extremely rare in *Ft* mutant ovaries leading us to conclude that these events culminated in premature ovarian failure.

Primordial germ cells of *Ft* mutant ovaries fail to proliferate in the mutant ovary. Whereas several of the factors that guide PGC and stimulate their division during migration have been identified, the supportive network within the gonad appears to be distinct and has yet to be

characterized. Initial proliferative events for PGC within gonads have been linked to signals from the Bmp family in both *Drosophila* and mice [196-199]. Of several Bmp ligands tested in the mouse, only Bmp7 had a transitory effect on germ cell proliferation within the early gonad, but this effect was specific to the XY gonad [196]. Expression of Bmp family members *Bmp2, -4, -5, -6*, or *-7* was not changed in *Ft* mutant ovaries supporting these results. Other factors, including fibroblast growth factor 9 (Fgf9) and Vasa also affected germ cell numbers only in XY gonads [200, 201]. Thus, we conclude that the *Ft* locus encodes genes that are essential for stimulating PGC proliferation once they inhabit the ovary. None of the genes within the *Ft* locus have been associated with cell cycle although there is still much to learn about these factors. Briefly, we know that the *IrxB* cluster genes are homeobox transcription factors and they could potentially transactivate a signaling molecule that supports PGC proliferation. *Ftm* encodes a protein associated with the ciliary body and hedgehog signaling and is discussed in more detail below [202]. This is the only gene that appears to be expressed within both somatic cells and PGC and therefore, may contribute directly to the PGC phenotype. *Fto* encodes a 2-oxoglutarate dependent DNA demethylase enzyme that is associated with energy metabolism [203]. The FTS (*Ft1, Aktip*) protein is related to ubiquitin-conjugating enzymes and binds directly with AKT to enhance its phosphorylation by promoting its interaction with the upstream kinase PKD1 [165]. *Ftm* deficient mice die around the time of birth [155]. $Fto^{-/-}$ mice are both viable and fertile whereas $Fts^{-/-}$ mice are viable with no obvious phenotype, but the reproductive status of females has not been tested [164] (U. Rüther, unpublished data).

It has been proposed that the first PGC to leave their site of origin at the base of the allantois are the first to arrive in the gonad and settle in the most anterior region. Those that follow migrate through to the posterior position of the gonad [204]. In *Ft* mutant embryos, PGC

numbers and migration were indistinguishable from wild type embryos at E9.5; however, by E12.5, PGC were generally lacking in the posterior region of the gonad, and the few germ cells that localized there were usually single cells or rarely contained within a germ cell nest of 2-3 oocytes. It is possible that the initial PGC pioneers were successful in reaching the gonad whereas the late migrating cohorts were lost. Alternatively, the paucity of PGC in the posterior gonad may be a consequence of defective proliferation and PGC inhabit this region simply as they multiply and passively occupy more space.

In the normal developing ovary, PGC enter meiotic prophase with all oocytes arresting in the diplotene stage of division by birth. However, meiotic germ cells have been observed in adrenal glands and the mesonephric region of male gonads in addition to reaggregated cultures within lung tissue demonstrating that female-specific support cells are not required for this event [205, 206]. The *Ft* mutant ovary contains maturing oocytes and somatic cells expressing female-specific markers; however, the structure is highly disorganized with few germ cell nests amid clumps of oocyte-less granulosa cells. Despite this chaos, oocytes progressed into meiosis within the *Ft* ovary supporting previous work describing oocyte independence from the surrounding somatic cells for this event. Oocyte maturation beyond meiosis, however, is severely affected in *Ft* mutant ovaries once the oocyte depends on the follicle as a unit.

Because *Ft* mutant embryos die early, we assessed follicle development after one, two, and three weeks transplantation under the kidney capsule of nude mouse hosts (equivalent to approximately P0, P7, and P14). Whereas wild type ovaries harbored several follicles that ranged from primary to pre-antral stages by three weeks after transplantation, *Ft* mutant ovaries contained gradually fewer follicles over time and these never progressed beyond the primary stage. These follicles also contained oocytes that were significantly smaller in diameter and

failed to grow as follicles matured up to the primary stage. These defects are likely explained by the degeneration of the granulosa cells, as marked by decreased expression of FOXL2 and MIS, and disintegration of the basal lamina that collectively signal the demise of the follicle unit at the expense of the oocyte. This particular phenotype is similar to that found in *Foxl2*$^{-/-}$ mice, which includes formation of primordial follicles that are surrounded by granulosa cells that fail to differentiate and proliferate, have disrupted basal lamina, and contain oocytes with retarded growth [179, 207]. However, *Foxl2*$^{-/-}$ ovaries do not share the early loss of PGC. Expression of *Foxl2* was significantly decreased within *Ft* mutant ovaries even though protein could still be detected at E12.5 and E12.5 plus two days culture (Fig. 2.15). Decreased FOXL2 expression was detected in ovaries later, after two weeks transplantation. Whether *Foxl2* expression is regulated by one of the genes within the *Ft* mutation or the decline of its expression merely marks abnormal granulosa cells remains to be determined.

None of the six genes missing in the *Ft* mutation have been associated with reproductive defects. To date, *Ftm*, *Fto, Irx3,* and *-5* have been associated with important developmental processes whereas the functions of *Irx6* and *Fts* remain unknown. Assessments of the phenotypes arising from functional studies of each gene provide few clues to explain the ovarian phenotype of the *Ft* mutant mouse. Only FTM, a ubiquitously expressed protein within the basal body of cilia that functions to help transduce the hedgehog signal [202, 208-210] could potentially explain some of the defects of the *Ft* mutant ovary. In the developing postnatal ovary, paracrine signaling between hedgehog-containing granulosa cells and neighboring granulosa and theca cells expressing the patched receptor has been suggested to contribute to granulosa cell proliferation and maturation of steroid-producing cells [211, 212]. None of the downstream components of the hedgehog pathway, including smoothened or patched receptors, were

expressed in oocytes of perinatal or adult ovaries [211, 212]. Therefore, the role of *Ftm* as a hedgehog mediator within the oocyte is an intriguing question. We are continuing to investigate effects of the *Ft* mutation on hedgehog signaling in addition to assessing the $Ftm^{-/-}$ mice to understand their contributions to ovarian development.

In conclusion, there are two primary events that occur within *Ft* mutant ovaries that ultimately result in ovarian failure. Different combinations of genes within the *Ft* mutation could cause each effect. The first is defective PGC migration into the gonad and failure of proliferation within that perhaps leads to an unsustainable numbers of oocytes inhabiting the ovary. This defect occurs between E11.5-14.5 making it less likely that the *IrxB* cluster genes, which do not reach their peak expression until mitotic proliferation is ending, contribute to this defect. *Irx6* expression is extremely low throughout development. However, *Ftm, Fts*, and/or *Fto* remain potential candidates for driving PGC proliferation at this time. The second is the disintegration of granulosa cells that occurs primarily after birth, when follicle units are maturing. *IrxB* cluster genes are more likely to contribute to this defect given their female-specific expression pattern; however, *Ftm, Fts*, and *Fto* may also be involved. All of the genes within the *Ft* locus are expressed within somatic cells and only *Ftm* is also expressed in PGC. These deficits contributed to defects within both cell types reinforcing the notion that two-way communication between these cell types is critical for normal follicular development. Ultimately, one or more of the genes within the *Ft* locus contributes to premature ovarian failure. Studies are currently underway to isolate the effects of each gene within the *Ft* mutation relative to gonad development.

Figure 2.1: Expression patterns in male and female gonads of the genes disrupted in the *Ft* mutant mouse.
(A-F) Quantitative PCR results for *Irx3*, *Irx5*, *Irx6*, *Ftm*, *Fto*, and *Fts*. Fold change was calculated relative to transcript levels of the female gonad at E12.0. Single asterisk indicates $p<0.01$; double asterisk indicates $p<0.001$.

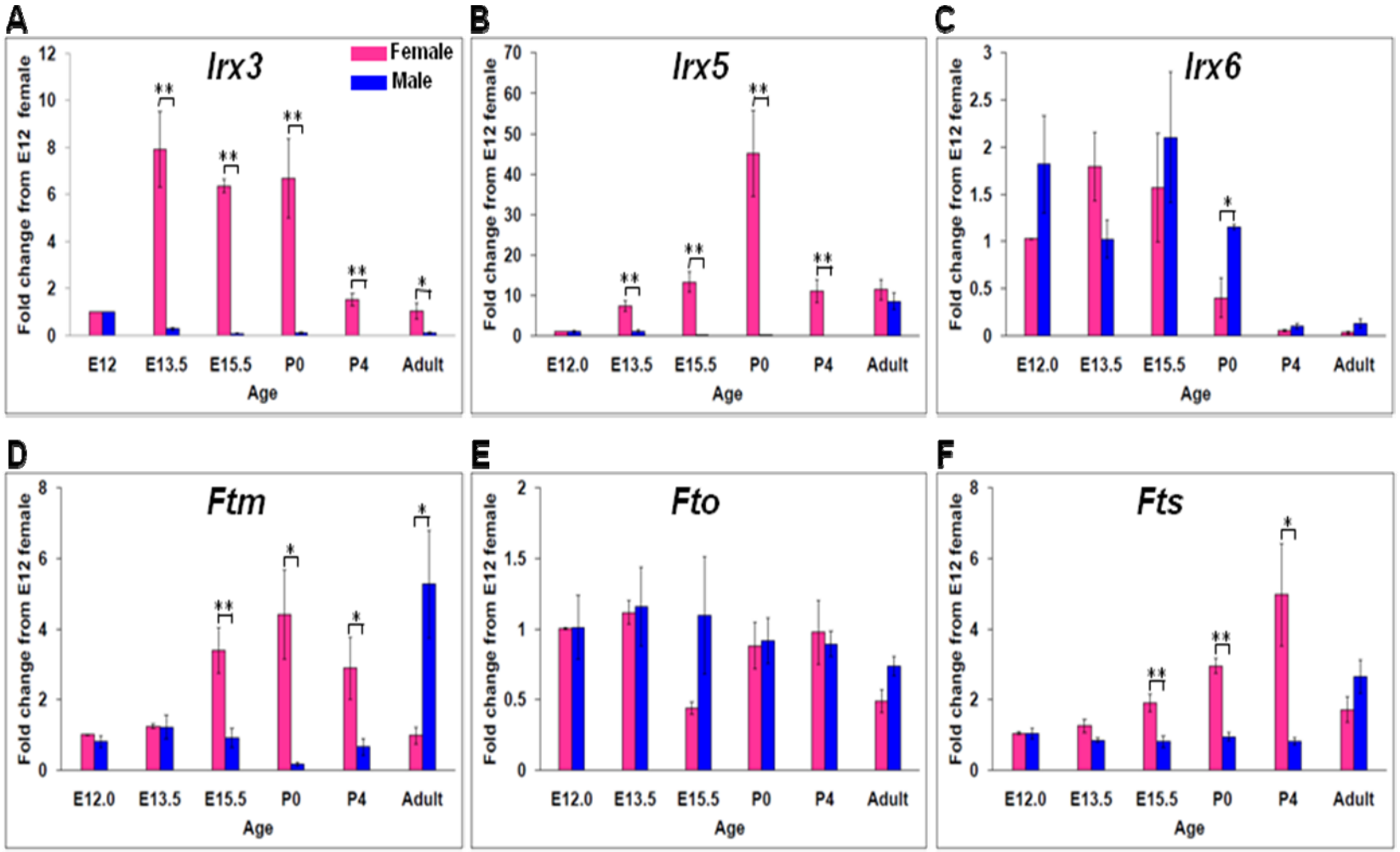


Figure 2.2: *Irx5-EGPF* activity is localized to somatic cells. (A) Double immunofluorescent staining for GATA4 protein (red) and EGFP (green) in an E13.5 *Irx5-EGFP* ovary section. (B) Double immunofluorescent staining for TRA98 protein (red) and EGFP (green) in an E13.5 *Irx5-EGFP* ovary section. GATA4 is a somatic cell marker and TRA98 is a germ cell marker.

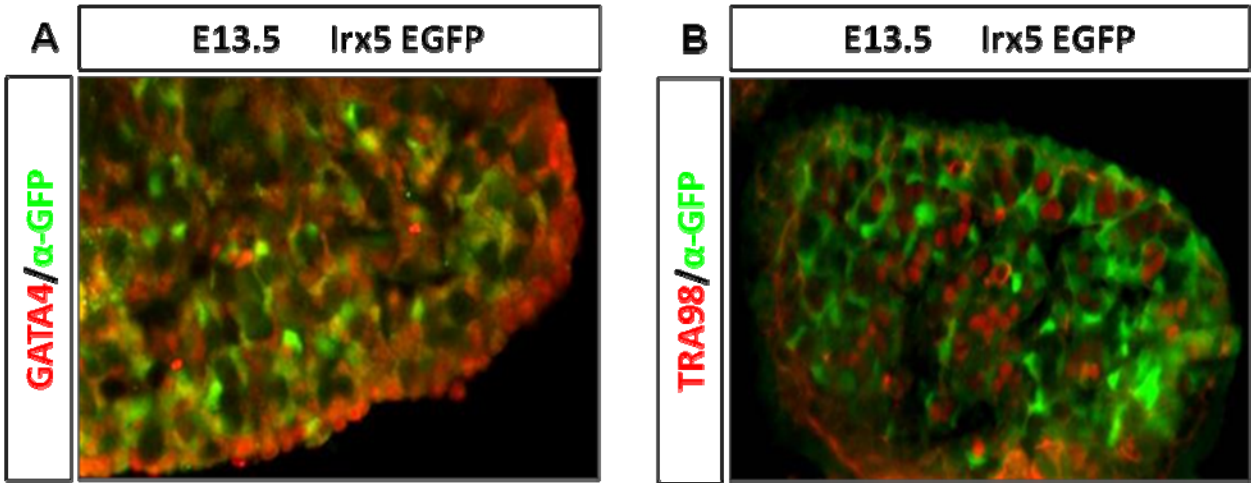


Figure 2.3: Quantitative PCR results showing expression of the Ft locus genes within female gonads harvested at E13.5 from embryos exposed to DMSO (Control, purple bars) or Busulfan (orange bars) treatment of pregnant females at E9.5. Single asterisk indicates p<0.0002; double asterisk indicates p<4.2202E-11.

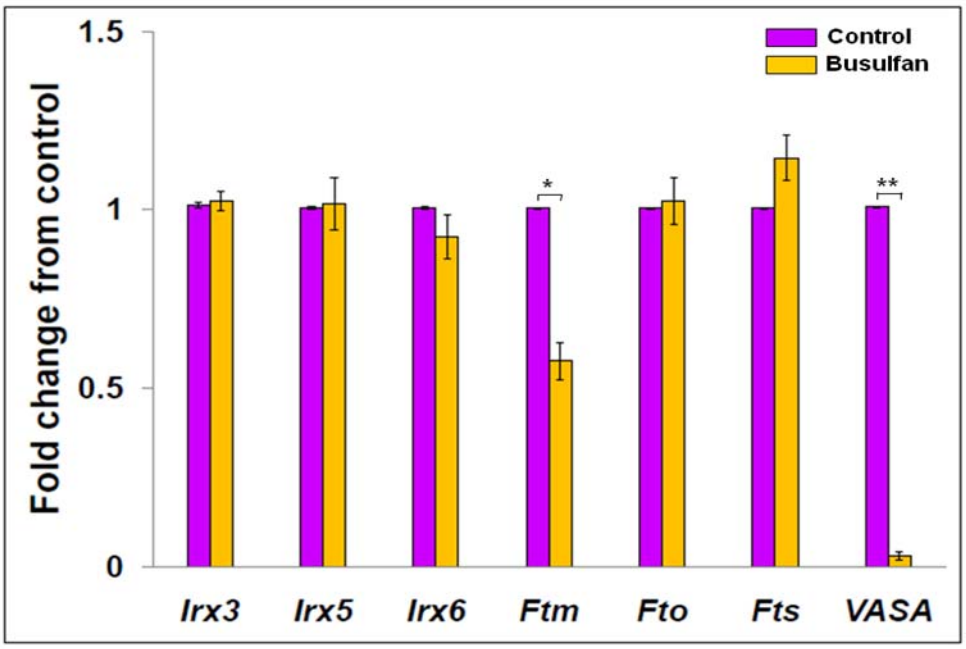


Figure 2.4: Ft mutant female gonads contain fewer germ cells. (A,B,B') Immunostaining for PECAM-1 protein in an E12.5 control (A) and Ft mutant ovary sections (B,B'). (C) Comparison of the number of germ cells. Asterisk indicates $p<0.001$.

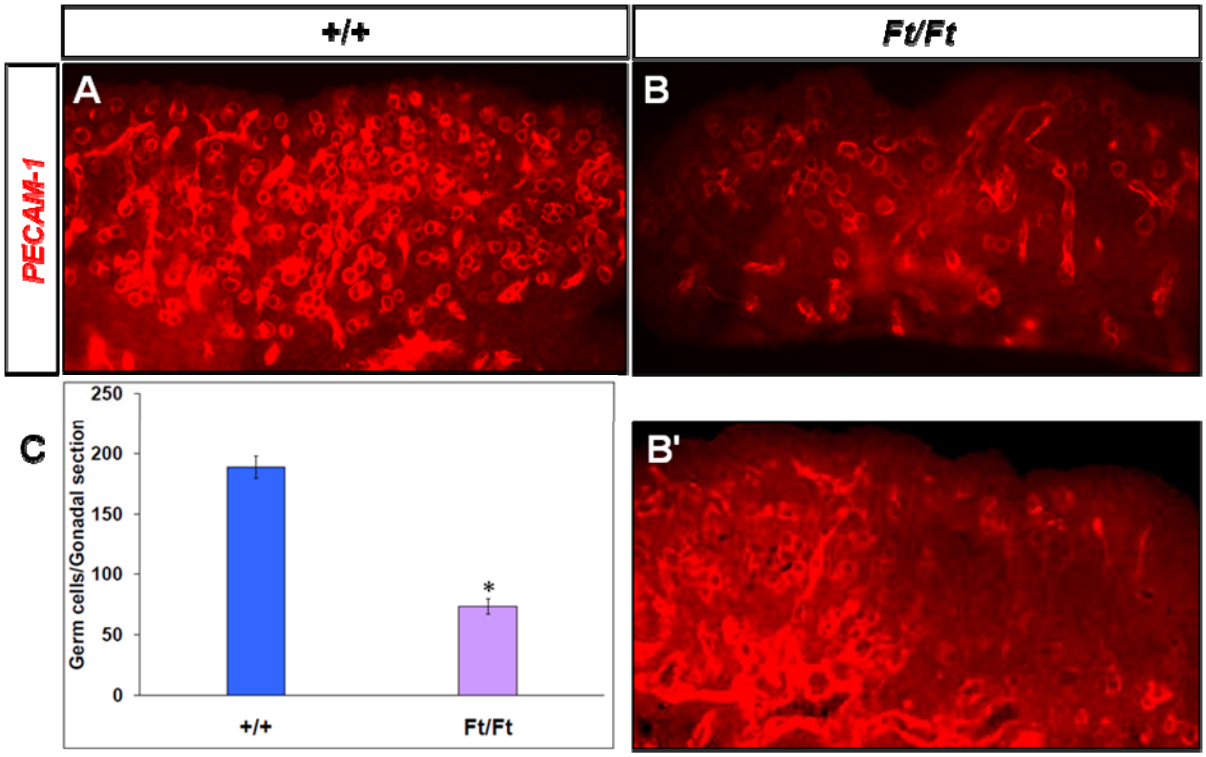


Figure 2.5: The reduced number of germ cells in E12.5 plus 2 days explants culture ovaries. (A,B) Double immunofluorescent staining for TRA98 protein (red) and FOXL2 protein (green). (C) The number of germ cells in E12.5 plus two days explants culture. Asterisk represents statistical difference from the wild type ($p<0.01$).

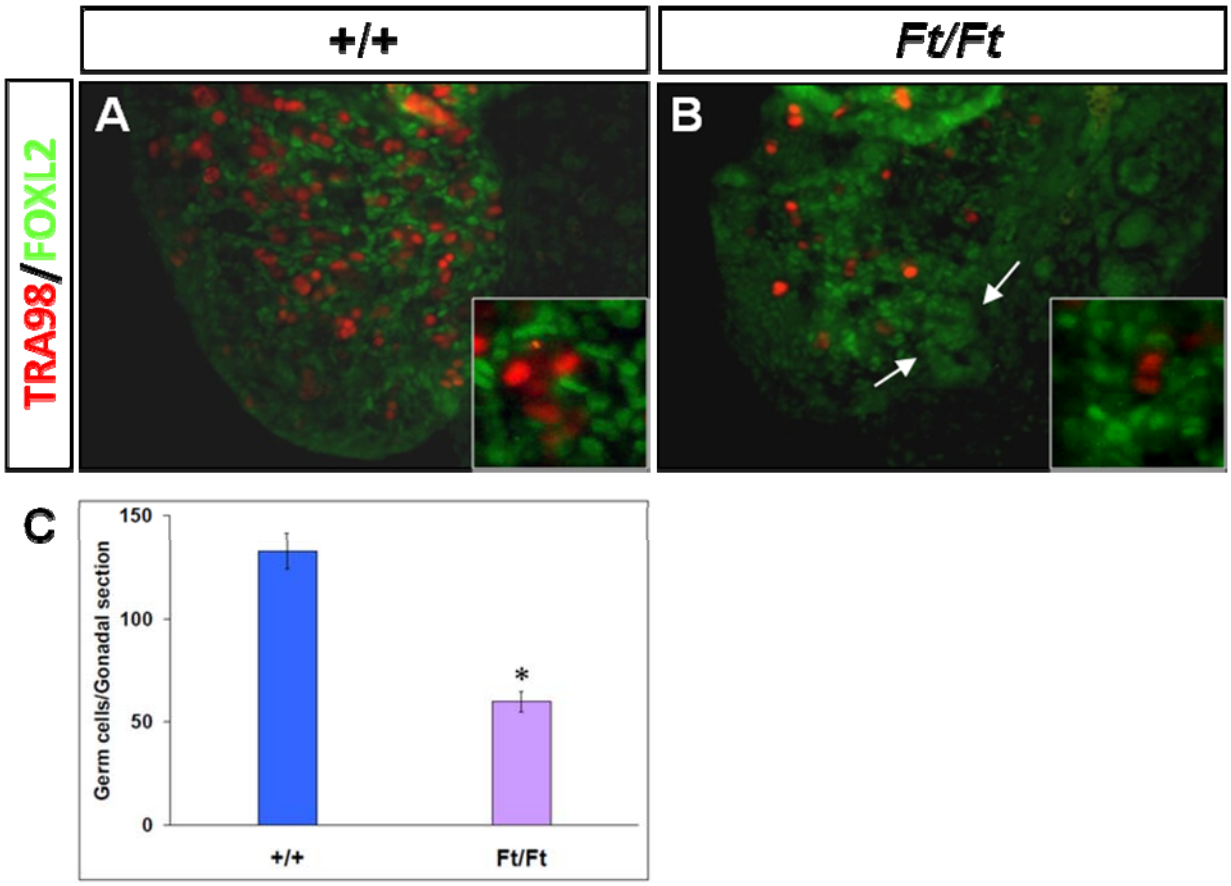


Figure 2.6: Vasa transcript levels are decreased in Ft mutant ovaries from E11.5 to E12.5 plus 2 days explant culture. Quantitative real time PCR was performed using primer for vasa and normalized to RNA of ribosomal subunit gene, 36B4 to obtain relative expression values. Relative expression of vasa was significantly decreased in Ft mutant ovaries (* $p<0.001$).

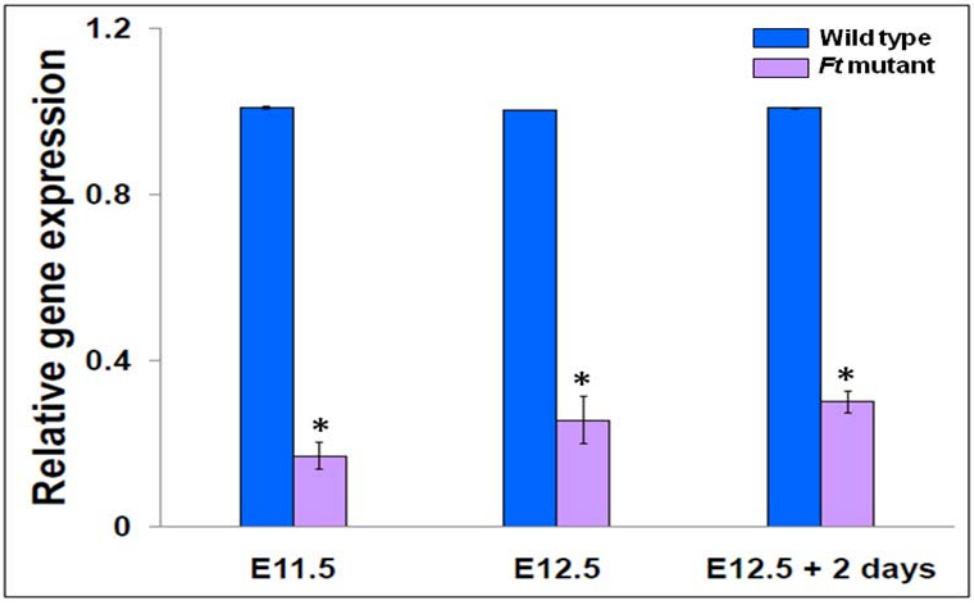


Figure 2.7: Clumped pre-granulosa cells in Ft mutant mice do not transdifferentiate into Sertoli cells. (A) Immunofluorescent staining for FOXL2 protein (green), SOX9 protein (red) and DAPI nuclear dye (blue) in the wild type of ovary tissues 1week after ovary transplantation. (B) Immunofluorescent staining for FOXL2 protein (green), SOX9 protein (red) and DAPI nuclear dye (blue) in the *Ft* mutant type of ovary tissues 1week after ovary transplantation. (C) A Testis for positive control for SOX9.

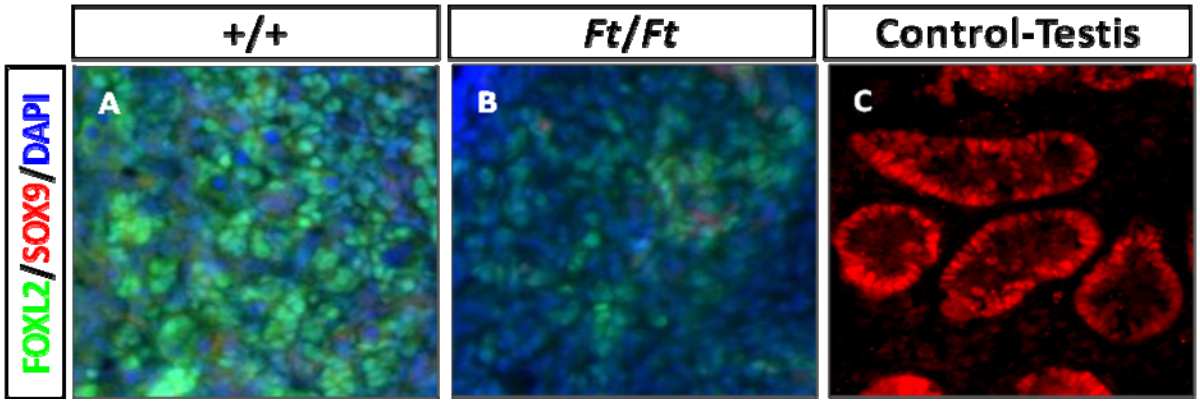
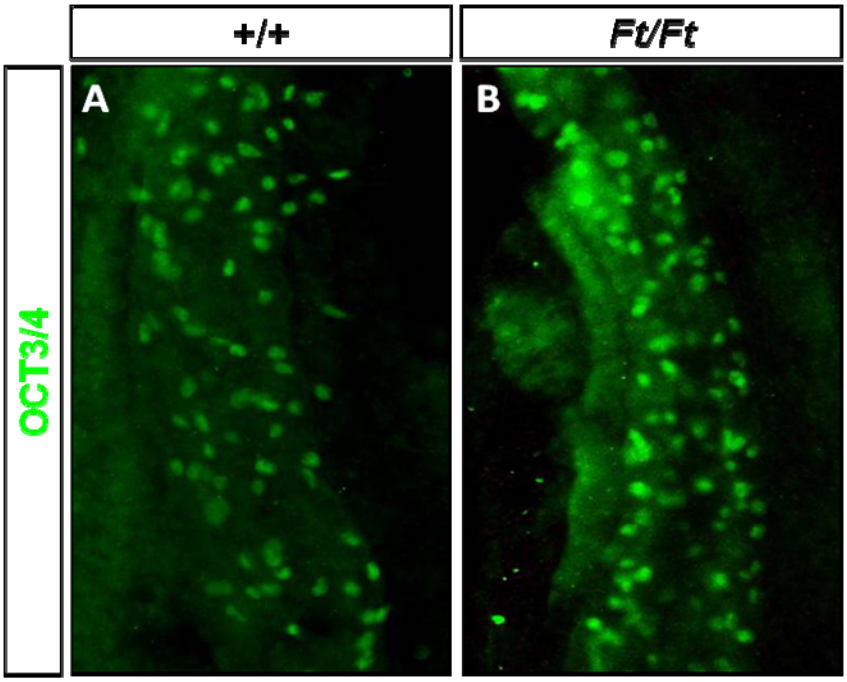


Figure 2.8: Whole mount immunostaining of primordial germ cells (PGC) in E9.5 embryos. (A,B) Immunofluorescent staining for OCT3/4 (green). (C) The number of PGC in wild and mutant type hindguts.



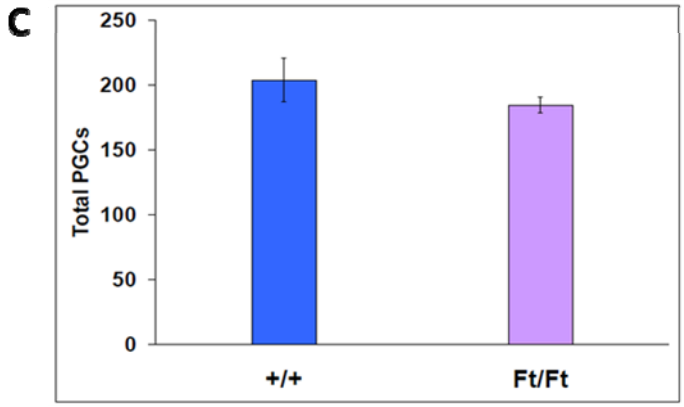


Figure 2.9: SDF-1 transcript levels in wild and Ft mutant ovaries

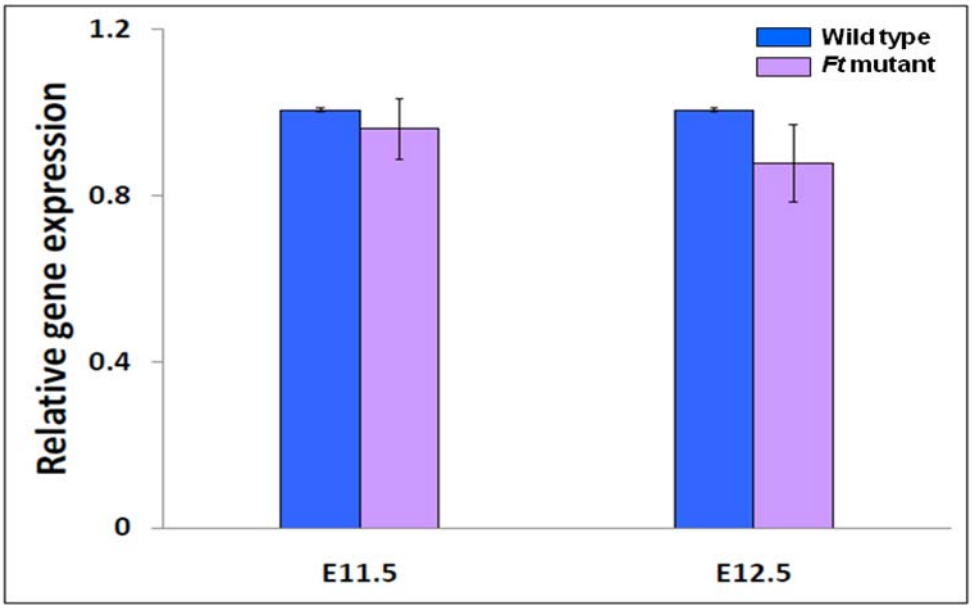


Figure 2.10: Germ cell proliferation is reduced in E12.5 *Ft* mutant ovaries. (A,B) Double immunofluorescent staining for PECAM-1 protein (green) and phosphorylated histone H3 protein (red) in E12.5 ovary sections. (C) The percentage of mitotic germ cells. Asterisk represents statistical difference from the wild type ($p<0.01$).

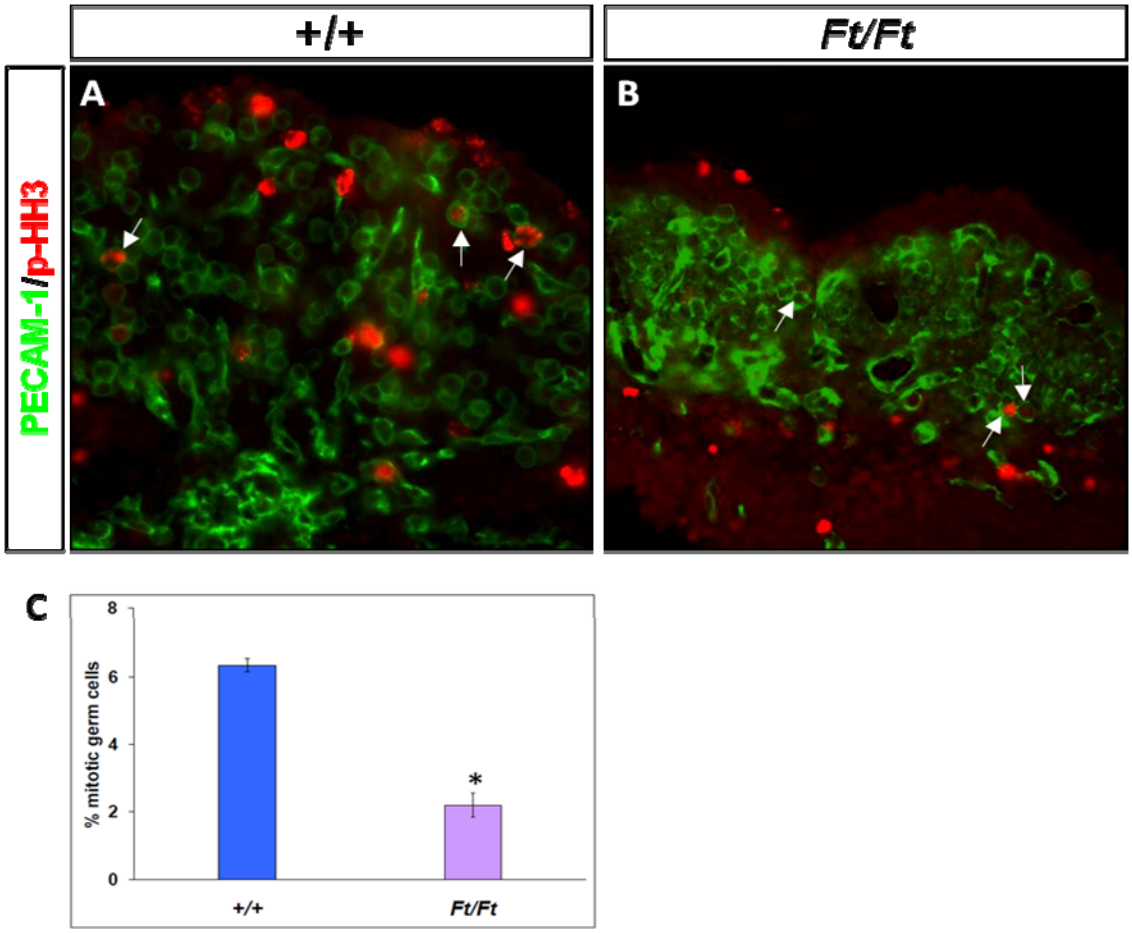


Figure 2.11: Germ cell proliferation is reduced in *Ft* mutant ovaries from E12.5 plus two days explants culture (A,B) Double immunofluorescent staining for TRA98 protein (red) and phosphorylated histone H3 protein (green) in E12.5 ovary sections. (C) The percentage of mitotic germ cells. Asterisk represents statistical difference from the wild type ($p<0.01$).

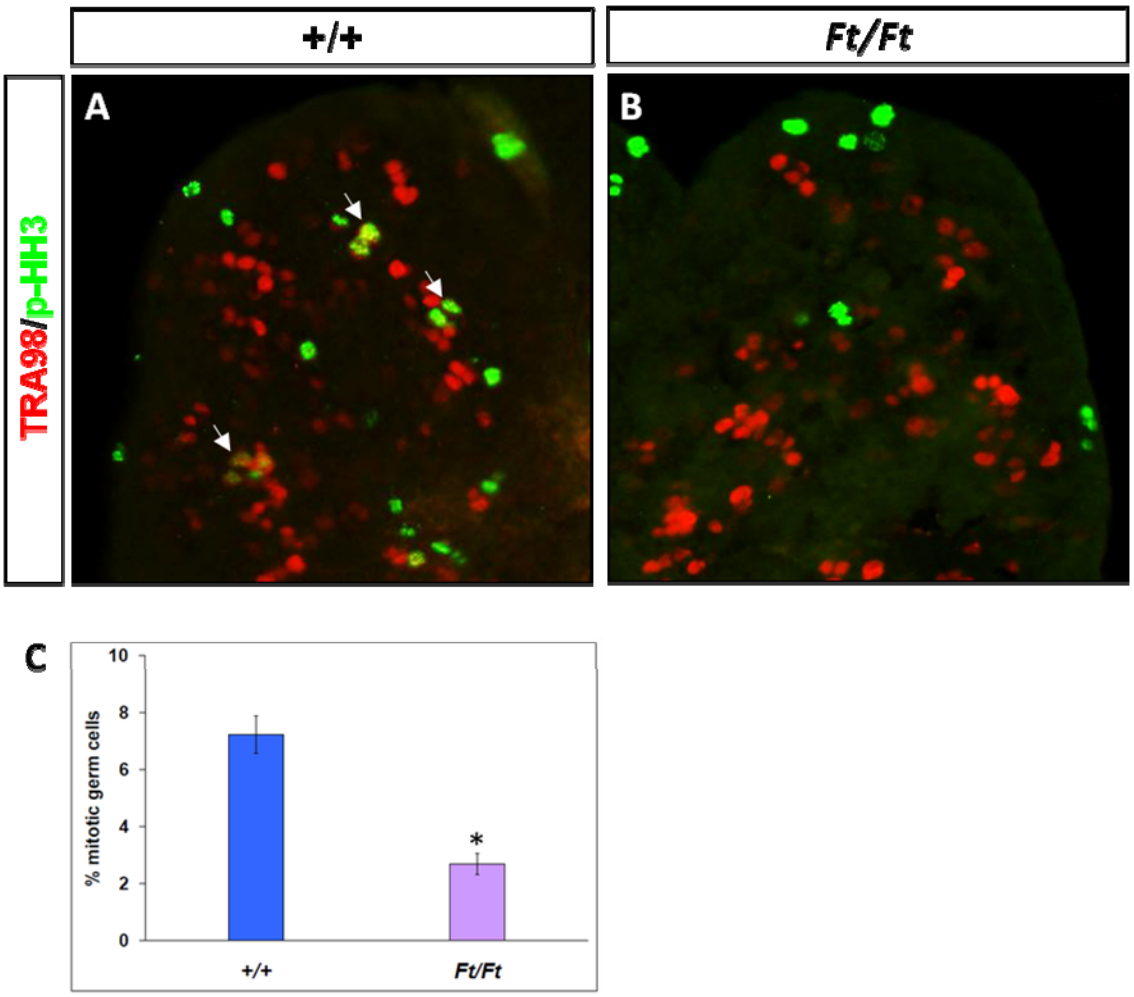


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Figure 2.12: Bmp family member transcript levels in wild *vs*. *Ft* mutant ovaries.

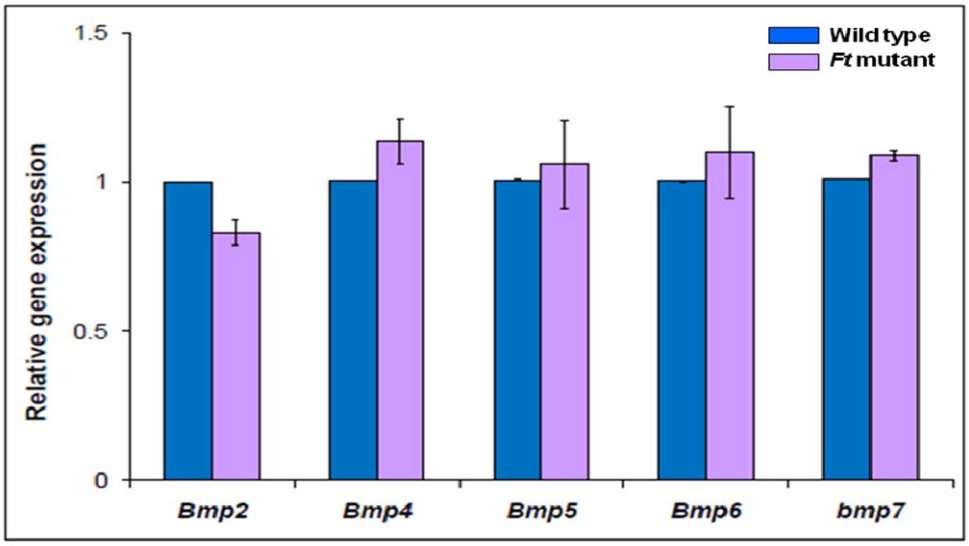


Figure 2.13: Follicle formation after ovary transplantation. Hematoxylin and eosin (H&E) staining of plastic or paraffin sections from wild type (A, C, E) and *Ft* mutant (B, D, F) ovary transplants. Black dotted outlines in (A, B) indicate the early primordial follicles. Arrow or arrowhead in (C) indicates a primary or secondary follicle, respectively. Arrows in (D) indicate abnormal follicles containing one or more oocytes surrounded by few fusiform granulosa cells. Arrow in (E) indicates a pre-antral follicle. Red dotted outline in (F) indicates a degenerated primary follicle.

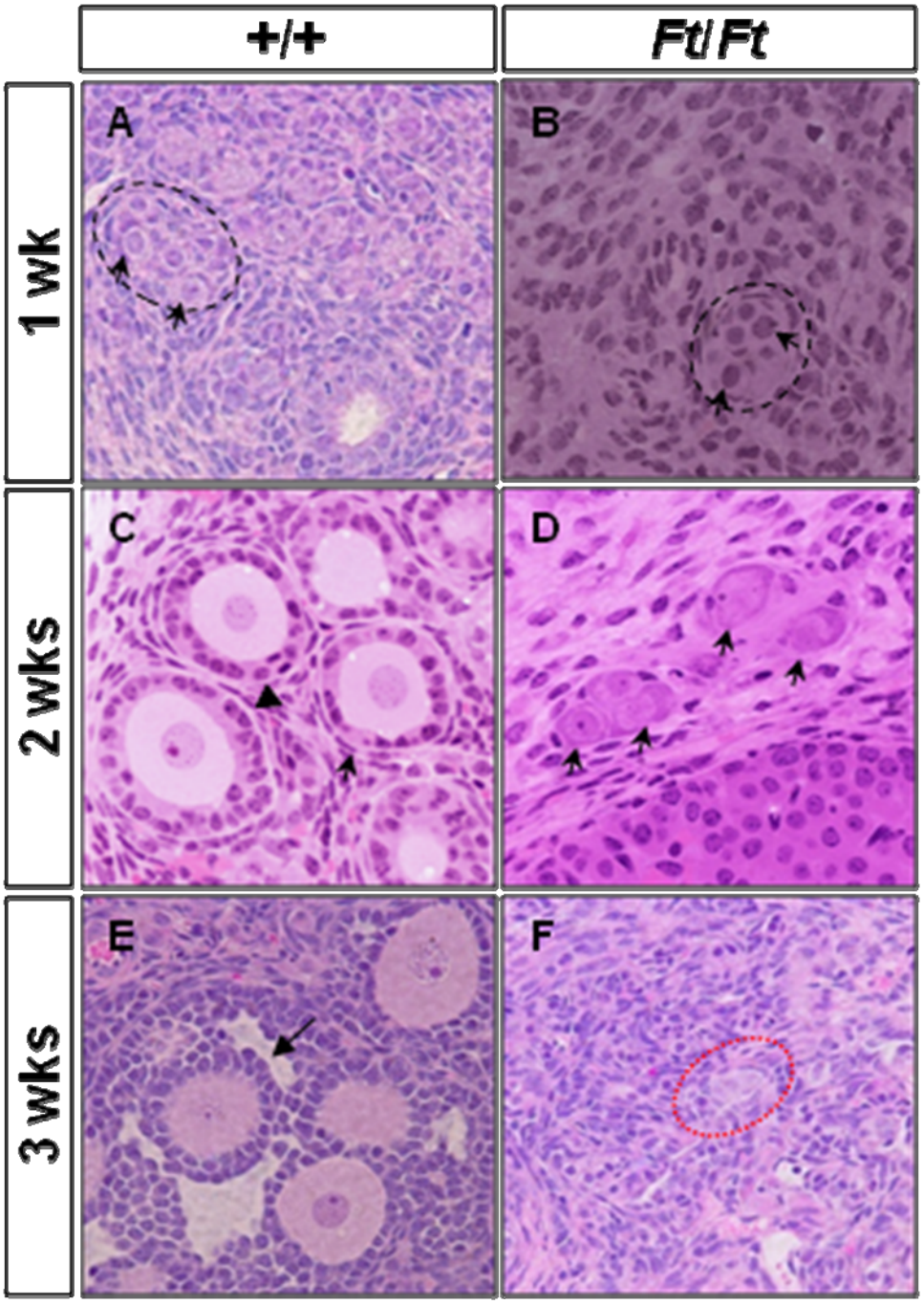


Figure 2.14: Few immature follicles survive in Ft mutant ovaries. (A,B) The meiosis marker, γH2Ax (red) is co-expressed in TRA98 labeled germ cells (green) in both wild type (A) and *Ft* mutant (B) ovaries transplanted for seven days (arrows). Inset shows magnified views of double-labeled oocytes. (C,D) Oocytes, highlighted by VASA (red), within the *Ft* mutant type ovary (D) are significantly smaller than age-matched wild type controls (C) after two weeks transplantation. Granulosa cells (MIS, green) surround oocytes (arrows) to form small follicles in addition to clumped clusters in Ft mutant ovaries transplanted for 2 weeks. (E-G) Ovaries from wild type (E) and Ft mutant (F,G) embryos were harvested after three weeks transplantation and assessed for follicle morphology. VASA (red) and MIS (green) expression illustrate several stages of follicle development, including pre-antral (arrow) follicles in the wild type (E) transplant ovary. *Ft* mutant ovaries contained very few follicles that appeared to be at either primordial (F) or primary (G) stages; these follicle are substantially smaller, note difference in magnification between E and F,G. (H,I) Ovaries subject to two weeks transplantation were assessed for expression of granulosa cell-specific markers, MIS (Red) and FOXL2 (Green). In contrast to the wild type ovary (H), the *Ft* mutant ovary (I) exhibited decreasing and irregular expression patterns of both proteins (dotted line outlines putative follicles, arrows highlight clumps of granulosa cells). (J,K) Ovaries were assessed for stromal structure after two weeks transplantation by examining laminin integrity. Laminin (green) and MIS (red) clearly outline primary follicles in the wild type transplanted ovary (J); however, laminin staining is disrupted along the borders of disorganized follicles of the mutant type ovary (arrows, K). Images show representative samples of at least 5 different biological replicates.

Figure 2.14 (cont.)

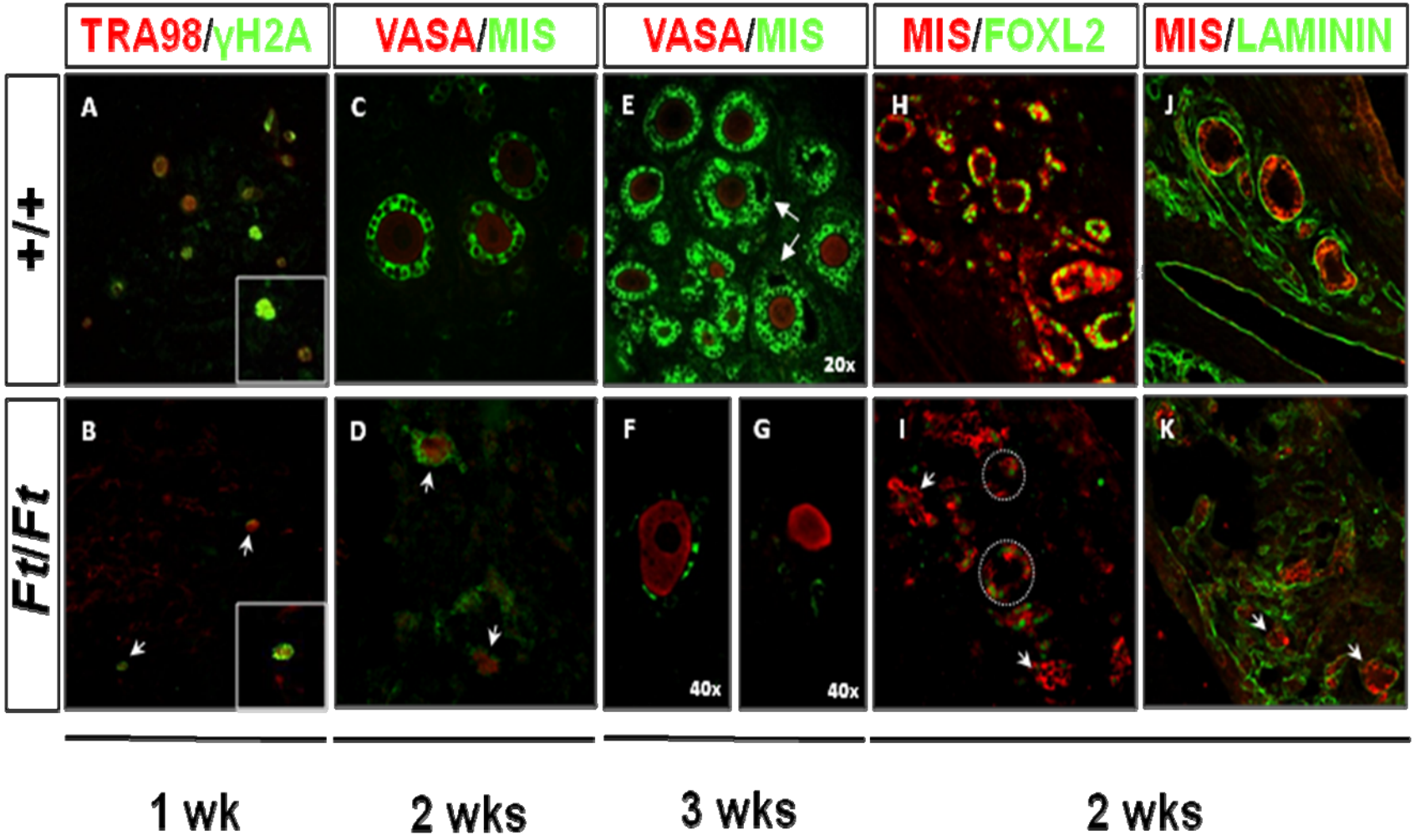
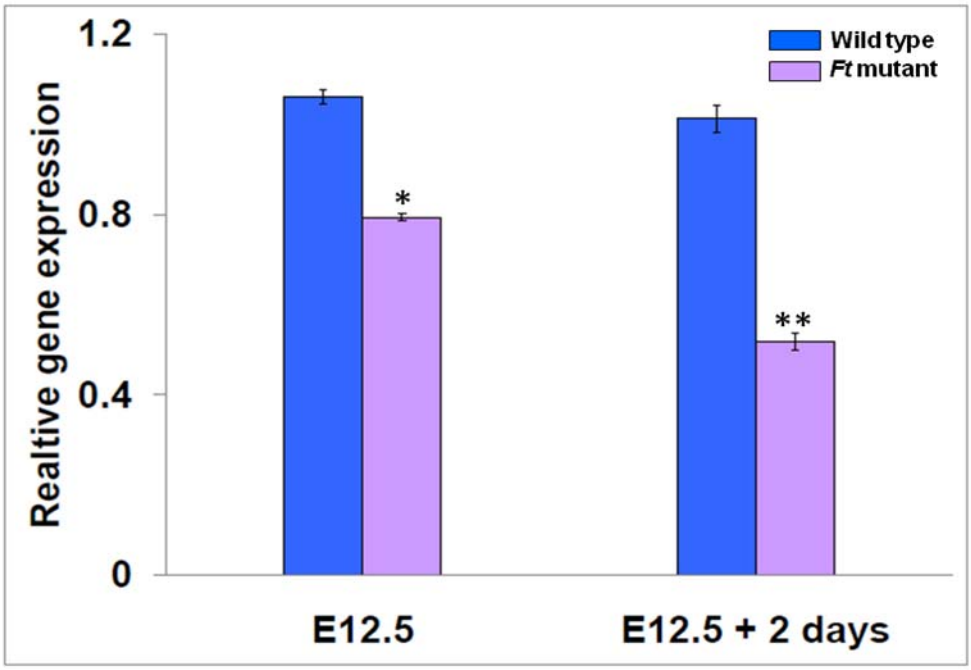


Figure 2.15: Foxl2 expression is decreased in Ft mutant ovaries at E12.5 and E12.5 plus two days explants culture. Quantitative real time PCR was performed using primer for Foxl2 and normalized to RNA of ribosomal subunit gene, 36B4 to obtain relative expression values. Relative expression of Foxl2 was significantly decreased in Ft mutant ovaries. (* $p<0.05$, ** $p<0.005$).



CHAPTER 3

REDUCED GERM CELL PROLIFERATION DURING EARLY DEVELOPMENT AND DISRUPTION OF SERTOLI CELL MORPHOLOGY DURING LATE TESTIS DEVELOPMENT IN FUSED TOES MUTANT MICE

3.1: ABSTRACT

Fused toes (*Ft*) mutant mice were used to investigate the function of six genes within the *Ft* locus: *Irx3*, *Irx5*, *Irx6*, *Fts*, *Fto*, and *Ftm* in testis development. These genes are present beginning at early stages of male gonadal development even though they are present at lower levels than in the female gonad. However, *Irx5*, *Ftm*, and *Fts* genes show increasing expression patterns in testes beginning at puberty. We previously demonstrated that the *Ft* locus genes are important for female gonadal development since its deletion causes defects in primordial germ cell migration from the hindgut to the genital ridge, germ cell proliferation which the gonadal ridge, and follicle formation. We concluded that the phenotype of the *Ft* mutant ovaries resulted from a failure of interactions between germ and somatic cells. Based on these results, our next question was whether the *Ft* locus is also required for normal testis development even though each of the six genes is expressed at lower levels in male versus female gonads. First, we found that *Ft* locus genes are required for early primordial germ cell migration and proliferation, similar to female gonads. This result suggests that the *Ft* locus genes regulate primordial germ cell migration and proliferation during early gonadal development in both sexes. Second, we found that fetal testis development is otherwise normal until birth. Third, during postnatal development, the *Ft* mutant testis shows a disruption of Sertoli cell architecture and a decrease in MIS expression. Our findings not only uncover new factors required for early primordial germ

cell migration and proliferation but also suggest the important function of the fused toes locus for Sertoli cell maturation.

3.2: INTRODUCTION

Primordial germ cells (PGC) originate from embryonal ectoderm. In the mouse embryo, PGC translocate by passive transfer to the hindgut starting at embryonic day 9.5 (E9.5). Then, the PGC join the gonadal ridge between E10.5 and E11.0. During early germ cell development and migration, several signaling factors have been shown to contribute to PGC survival and/or proliferation. Both white spotting (*W*) and Steel (*Sl*) mutant mice contained few germ cells [213-215]. The W locus encodes c-kit, a tyrosine kinase receptor, on the surface of germ cells [216]. Its ligand, stem cell factor (kit ligand/SCF), is encoded by the *Sl* locus and is expressed by the hindgut and dorsal mesentery along the germ cell migratory pathway [217]. Another ligand and receptor pair, stromal cell-derived factor-1 (SDF-1) and CXCR4 are expressed by somatic cells of the gonad and germ cells, respectively. The SDF-1/CXCR4 pathway is also necessary for PGC migration and survival [218]. Together, these studies show that interactions between germ and somatic cells are very important for PGC migration, survival, and proliferation. However, the cellular and molecular basis of these mechanisms is not well understood.

Once male PGC have arrived in the gonad, they interact with epithelial and mesenchymal cells of the gonad to form testicular cords [219]. Sertoli cells are the first somatic elements to differentiate in the testis and they play a central role in testis differentiation [220]. Sertoli cells proliferate actively before birth and continue to divide until 2 weeks after birth in mice [221, 222]. Thereafter, the number of Sertoli cells per testis is considered stable and they begin to differentiate [223]. Within the seminiferous epithelium of the testis, Sertoli cells

produce many soluble factors necessary for germ cell survival and proliferation, for basement membrane components, and for maintaining the tubule structures within which germ cells differentiate [224]. SCF, basic fibroblast growth factor (bFGF), and leukemia inhibitory factor (LIF), all of which are produced by Sertoli cells, seem to be particularly important for the survival and proliferation of PGC, gonocytes, and type A spermatogonia [225-230], whereas transforming growth factor beta (TGF-β) has been implicated in germ cell differentiation at later stages [231-233]. Additionally, the A_{single} spermatogonia located in the basal compartment of the seminiferous epithelium and in direct contact with Sertoli cells and the basement membrane, are maintained by the glial cell line-derived neurotrophic factor (GDNF) [234]. Proper relationships between Sertoli cells and germ cells are required for normal testis development and spermatogenesis.

The *Ft* mutation is a 1.6-Mb deletion on mouse chromosome 8 that eliminates six genes including the Iroquois homeoboxB (*IrxB*) cluster and three other genes, *Ftm, Fto and Fts* [154]. Heterozygote animals develop fused toes in the forelimbs and hyperplasia of the thymus. Both defects have been attributed to abnormal programmed cell death [156]. In the homozygous state, *Ft* mutant mice suffer severe abnormalities in brain, spinal cord, craniofacial, heart, and limb development and embryos die between E10.5 and E14.5 [121, 235-239].

In Chapter 2, we reported that the *Ft* locus genes are present in both male and female gonads with different expression patterns. PGC failed to migrate to ovaries in *Ft* mutant embryos and PGC proliferation was dramatically reduced within the ovary. In addition, the few follicles that formed failed to progress past the primary follicle stage.

Here we report that *Ft* mutant male gonads have significantly reduced germ cell numbers because of failed germ cell proliferation during embryonic gonadal development

between embryonic days 11.5 (E11.5) and E14.5. Otherwise, the testis develops normally until after birth. These results support previous findings that suggest that the presence of germ cells are not required for the formation of testis cords [240]. However, we found that Ft mutant Sertoli cells lose their function during the transition from proliferation to differentiation stages which occurs around 2 weeks after birth. The lost function of Sertoli cells induces failure of organization of the few remaining spermatogonial cells which are normally located in the basal compartment of the seminiferous epithelium in contact with Sertoli cells.

3.3: MATERIALS AND METHODS

3.3.1: Animals

Heterozygote female and male *Ft* mutant mice were paired together and checked for the presence of a vaginal plug the next morning. The day when the vaginal plug was detected was considered 0.5 day of gestation, or E0.5. Gonads were collected at E11.5 or E12.5 because homozygous *Ft* mutant embryos died between E10.5 and E14.5 depending on background strain; our colony was maintained in a C3H background. The gonads were cultured in DMEM with 10% FBS or transplanted into the kidney capsule of a nude mouse host. For genotyping, embryo tail bits were digested in 50 mM sodium hydroxide and subjected to standard PCR using gene specific primers: Ft:5'-GTCCTTTCTCCATGGGTATG-3', Wt: 5'-GTGGAACCCTTCTGTACATG- 3', Ft/Wt: 5'-CTGAAAGGTTGTACTGAGCC-3', which allowed discrimination between the three genotypes. For sex genotyping, primers 5'-TGCAGCTCTACTCCAGTCTTG-3' and 5'-GATCTTGATTTTTAGTGTTC-3' were used to detect the presences of the *Sry* gene. All procedures described were reviewed and approved by the Institutional Animal Care and Use Committee at University of Illinois and were performed in

accordance with the Guiding Principles for the Care and Use of Laboratory Animals. All experiments were performed on at least five animals for each genotype.

3.3.2: RNA extraction and real-time PCR

RNA was prepared from pooled individual gonads (separated from mesonephros) from E12.0-E15.5 gonads, and testes using the RNeasy Micro Kit (Qiagen). Adult testes were collected from 6-7 week-old mice. All quantitative real-time PCR assays were carried out using the SYBR Green I Kit (Applied Biosystems) as previously described [183]. The relative expression level for each sample was determined in the same run and was expressed as the ratio of the quantity of RNA of interest to that of a control RNA (36B4). Gene specific primers were designed using Primer Express; primer sequences are shown below (Table 3.1).

3.3.3: Germ cell depletion by busulfan treatment

Female pregnant mice were administered an intraperitoneal injection of 100 μℓ Busulfan solution (16 mg/ml in 50% DMSO) or 50% DMSO (control) at E9.5 as previously reported [183]. Testes were harvested from embryos of the treated females at E13.5 for RNA isolation and quantitative real-time PCR.

3.3.4: Testis transplantation

Testis were harvested at E12.5 from wild type or *Ft* mutant embryos and then transplanted underneath the kidney capsule of castrated nude mice of at least 8 weeks of age (Charles River Laboratory). Castration was performed 10-14 days before transplantation surgery. For kidney capsule transplantation, a dorsolateral incision was made into the skin and peritoneum to expose the kidney. A small incision was made into the kidney capsule and a pair

of wild type or *Ft* ovaries was inserted under the capsule. The kidney was returned to its normal anatomical position. The body wall was sutured, and the skin was closed with wound clips. Grafted testes were recovered 1 week after transplantation. To analyze long term development, harvested grafted testes were transferred under the dorsal skin after one week under the kidney capsule. Tissues were harvested 1 or 2 weeks after transfer under the skin.

3.3.5: Immunohistochemistry

Testes and transplanted tissues were fixed in 4% paraformaldehyde in PBS at 4℃ overnight and then rinsed three times in PBS for 5 min each. Then samples were put through a sucrose gradient (10%, 15% and 20%) followed by incubation in 1:1 20% Sucrose and OCT-freezing medium (Tissue-Tek) at 4℃ overnight. The samples were embedded in a 1:3 20% sucrose and OCT cocktail and cut into 10㎛ frozen sections. Primary antibodies were as follows: goat anti-MIS (1:250; Santa Cruz Biotechnology), mouse anti-Tra98 (1:250; Cosmo Bio), rat anti-mouse CD1 (1:100; BD Biosciences), rabbit anti Phospho-Histone H3 (1:250; Cell signaling), and rabbit anti-LAMININ (1:250; Sigma-Aldrich). All secondary antibodies (FITC or Rhodamine-conjugated donkey anti-rabbit, goat, and mouse antibodies) were obtained from Jackson Laboratory and used at a dilution of 1:300. Images were collected on a Nikon E600 microscope and processed using Adobe Photoshop.

3.3.6: Histology

Samples were fixed with 4% paraformaldehyde (PFA) in PBS overnight at 4℃, processed through a graded ethanol series, embedded in paraffin, sectioned to 5㎛, deparaffinized, rehydrated and stained with hematoxylin and eosin. Slides were mounted using Permount. Plastic sections were used to study the ultrastructure of transplanted testis tissues.

Testes were fixed in 4% glutaraldehyde overnight and kept in 2% glutaraldehyde at room temperature or 4℃. Tissue processing and staining (Toluidine blue/basic Fuchsin staining) were performed in The Center for Microscopic Imaging, College of Veterinary Medicine, University of Illinois. The procedures are based on previously described methods [188].

3.3.7: Oct4 whole-mount embryo immunohistochemistry

E9.5 embryos were fixed in 4% paraformaldehyde (PFA) and then washed in PBS. Embryos were blocked in PBSST (PBS/0.5% Trition X-100 with 5% donkey sera, 2x1 hour washes) and incubated overnight at 4℃ in PBSST with goat anti-Oct-3/4 (Santa Cruz Biotechnology; 1:100 dilution) antibody. The following day, the embryos were washed in PBSST (3X 1 hour) at room temperature and then incubated overnight at 4℃ in PBSST with doney anti-goat-Rhodamine-conjugated secondary antibody (Jackson Laboratory; 1:300 dilution). The embryos were washed in PBSST (5X 1 hour) and then mounted with Vectashield mounting media (Vector Laboratories, Burlingame, CA).

3.3.8: Germ cell counts

Total numbers of PGCs in E9.5 hindguts were counted using a fluorescence microscope after staining for Oct4.

Germ cells of E12.5 gonads and E12.5 plus 2 days cultured gonads were counted using at least 5 different biological samples for each genotype using a previously described method [189]. Briefly, gonads were stained using antibodies against PECAM-1 or TRA98 and imaged using a fluorescence microscope. Longitudinal sections from the central third of each testis were selected. For each testis, all PECAM-1 or TRA98-positive cells were counted. To quantify the percentage of germ cell proliferation for E12.5 gonad sections the number of pHH3 positive

germ cells were divided by the total number of germ cells (PECAM-1 positive germ cells), or for E12.5 plus 2 day cultured gonad sections the number of pHH3 positive germ cells were divided by the total number of germ cells (TRA98 positive germ cells).

3.3.9: Statistical analysis

Statistical differences were determined using two-tailed Student's t test comparisons.

Table 3.1: PCR primers for real-time PCR (written 5' to 3')

Gene	Forward primer	Reverse primer
Irx3	CGCCTCAAGAAGGAGAACAAGA	CGCTCGCTCCCATAAGCAT
Irx5	GGCTACAACTCGCACCTCCA	CCAAGGAACCTGCCATACCG
Irx6	AGCACATCCCAGTTTCTGGTGTCT	ACAGCAAAGAGTAGAGGCAGAGGT
Fts	TTCACCCACTAGTTGATCCCACCT	ATGGTTATGGTTCCGCCTCCACTT
Ftm	TGCAGCGTGTCAGTTGAGATTCCA	TATCCTAATGCCTTTGCCCGCTCA
Fto	TGCGAAGGCTCTGAGGATGAAAGT	AATCCTGGTGTCTCGATGTCCCAA
SDF-1	GAGAGCCACATCGCCAGAG	TTTCGGGTCAATGCACACTTG
Vasa	GAGATTGCCTTCAGTACCTATGTG	GTGCTTGCCCTGGTAATTCT
36B4	CGACCTGGAAGTCCAACTAC	ATCTGCTGCATCTGCTTG

3.4: RESULTS

3.4.1: Expression profile of *Ft* locus genes in male gonads

We analyzed expression patterns of each gene, *Irx3*, *Irx5*, *Irx6*, *Ftm*, *Fto*, and *Fts* in wild type male testes from E12.0 to adult (Fig. 3.1). Each gene was present in the developing testis. During embryonic testis development, *IrxB* cluster genes, especially, *Irx3* and *Irx5* decreased until birth (Fig. 3.1A-B), and the other four genes, *Irx6*, *Ftm*, *Fto*, and *Fts*, were present without changing (Fig. 3.1C-F). In adult testes, however, three genes, *Irx5*, *Ftm*, and *Fts* showed high expression levels (Fig. 3.1B,D,F). Therefore, these three genes may have more important functions during adult testis development.

Next, we investigated whether these genes were expressed in germ cells, somatic cells or both (Fig. 3.2). Pregnant mice were injected with busulfan which caused loss of most PGC in the embryos. The Vasa gene was used as a reference gene to confirm the number of germ cells in wild type and busulfan treated groups. Unlike results from female gonads, all six genes showed no differences between the control and the busulfan treatment groups (Fig. 2.3). *Ftm* expression was decreased in female gonads after exposure to busulfan, but this difference was not detected in male gonad samples (Fig. 3.2). These results indicate that the expressions of these six genes do not depend on germ cells, and that *Ftm* might have different functions in male and female gonads (Fig. 2.3; Fig. 3.2).

3.4.2: *Ft* mutant testis contains few germ cells

Immunofluorescence staining was performed with PECAM-1, which is a germ cell and endothelial cell marker, in E12.5 gonads (Fig. 3.3A-B). Significantly fewer germ cells were observed in the Ft mutant testes (Fig. 3.3A,C). Similarly, Ft mutant testis gonads were observed with significantly reduced TRA98- positive cells in E12.5 plus 2 days cultured gonads (Fig.

3.4A, B). In addition, Vasa transcripts were assessed between wild type and mutant type gonads using quantitative real time PCR at E11.5, E12.5 and E12.5 plus 2 days culture. Vasa transcripts were decreased significantly in *Ft* mutant testes at each time point (Fig. 3.5). These results show that the *Ft* mutant testes have reduced numbers of germ cells from E11.5, suggesting that at least one of the *Ft* locus six genes affects, directly or indirectly, germ cell development.

To investigate whether primordial germ cells migrate and proliferate normally in the *Ft* mutant mice, Oct3/4 positive PGC were counted within the hind gut at E9.5. PGC numbers were not statistically different between wild type and *Ft* mutant embryos (Fig. 3-6A-C) suggesting that *Ft* mutant embryos have normal PCG specification, migration, and proliferation until E9.5. Next, we assessed the SDF-1 gene expression pattern, which is known as a key gonad-derived factor to attract germ cell migration into gonads. No difference was detected in SDF-1 transcript levels at either E11.5 or E12.5 gonads between the wild and mutant gonads even though fewer germ cells were present (Fig. 3.7).

3.4.3: Reduced germ cell proliferation in *Ft* mutant male gonads

Primordial germ cells migrate from the hindgut to the genital ridges around E10.5 in mice. XY germ cells continue to keep proliferating until E13.5 and then undergo mitotic arrest. We assessed germ cell proliferation and cell death to determine the cause of the decrease in germ cell numbers within *Ft* mutant testes. Testes from E12.5 and E12.5 plus 2 days culture were stained with Caspase3 and a germ cell marker to assess apoptosis. No difference in apoptosis was detected between wild type and *Ft* mutant testes. Next, we stained gonads with p-HH3 and PECAM-1 or TRA98 to assess the numbers of proliferating germ cells. The average percentage of germ cells positive for p-HH3 staining was significantly lower in *Ft* mutant gonads at both stages (Fig. 3.8A-B; Fig. 3.9A-B, arrows). In addition, wild type germ cells were present as

clusters with a few germ cells (cyst formation) (Fig. 3.9A, white dotted line), but most of the *Ft* mutant type of germ cells were present with single or two germ cells (Fig. 3.9B, white dotted line). Generally, male premeiotic germ cells develop within formed cyst in the mouse and other vertebrates [241, 242]. Our results suggest that reduced germ cell proliferation results from failure of germ cell cyst formation in *Ft* mutant testes. BMP family members are known to contribute to embryonic germ cell proliferation in mice, especially, BMP7 [195]. We investigated expressing five BMP family genes (Bmp2, 4, 5, 6, and 7) but could not find any difference in transcript levels between wild type and mutant testes (Fig. 3.10). These data suggest that the deficiency of proliferation in *Ft* mutant gonads is involved through other factors that may include members of the *Ft* locus. The mechanism behind this defect must be further studied.

3.4.4: Normal testis formation despite reduced germ cell numbers in *Ft* mutant mice

To assess further development, *Ft* testes or Wt testes from E12.5 were transplanted underneath the kidney capsule of castrated nude mouse hosts for one week (≈ P0). The morphology of grafted tissue between *Ft* mutant and wild type testes was similar (Fig. 3.11A-B). Although they both have lower numbers of germ cells compared to age matched non-grafted tissues, both testes contained normal Sertoli cells and interstitial cells. Double immunofluorescence staining for MIS and CYP17 was performed to observe Sertoli and Leydig cells. No differences were detected between the wild type and mutant testes at this stage (Fig. 3.12A-B). In addition, we examined the expression of Laminin-1 and TRA98 to detect basal lamina formation in relationship to germ cells. Basal lamina were observed clearly in both wild type and mutant testes, and germ cells were present inside the testicular cord (inside of basal lamina) (Fig. 3.12C-D). Together, these results suggest that Leydig, peritubular myoid, and

Sertoli cell lineages of *Ft* mutant testes can develop until birth, even though there are few germ cells in the early stage. These results support previous findings that claimed that germ cells are not required for normal testis formation in mice [240].

3.4.5: Failure of Sertoli cell differentiation in *Ft* mutant mice

To assess testicular development that would theoretically occur after birth, we examined testes that were transplanted under the kidney capsule for one week followed by transplantation under the dorsal skin of a new mouse host for an additional two weeks. Testes were transferred under the dorsal skin to allow testis development in an environment that has a lower temperature, which mimics that of the scrotum. These testes, which were transplanted for a total of three weeks, were considered to be age matched to 2 week-old testes. Testis morphology was observed by hematoxylin and eosin (H·E) staining (Fig. 3.13A-B). Most of the peritubular myoid cells and the cytoplasm of Sertoli cells were disrupted in many *Ft* mutant testes (Fig. 3.13B, arrow). To assess each cell type, samples of wild and *Ft* mutant type testes were examined by double immunofluorescence staining using specific markers: MIS for Sertoli cells, Laminin-1 for basement membrane, and TRA98 for germ cells (Fig. 3.14A-D). We found that the MIS expression pattern revealed dramatic decreases in *Ft* mutant Sertoli cells (Fig. 3.14B). In wild type gonads, most of the spermatogonial cells were located next to the basement membrane compartment and in direct contact with Sertoli cells (Fig. 3.14C). However, in the *Ft* mutant testis, few germ cells were present next to the basement membrane compartment and many germ cells were still located in the middle of the seminiferous epithelium (Fig. 3.14D). It is possible that failure of Sertoli cell maturation disrupted the normal interaction with germ cells and movement of germ cells to the basement membrane. In addition, the dysfunction of Sertoli cells induced failure of basement membrane formation.

These findings suggest that some genes of the *Ft* locus are essential for normal testis development. Also, more studies are required for elucidation of the exact mechanism.

3.5: DISCUSSION

Our results show that *Ft* mutant male testes display the following abnormalities: 1) reduced germ cell numbers and germ cell proliferation during early gonadal development and 2) abnormal Sertoli cell maturation in the postnatal testis. These data support that the presence of the *Ft* locus is necessary for normal testis development.

3.5.1: How is the *Ft* locus related to germ cell development?

In Chapter 2, we proposed that the *Ft* locus is required for normal germ cell migration and proliferation during early female gonadal development. Interestingly, we found that this locus is also necessary for male germ cell migration and proliferation. we found that *Ft* mutant PGC showed normal development within the hindgut until E9.5. This suggests that *Ft* mutant PGC are normally specified and can migrate and proliferate within the hindgut until at least E9.5. Moreover, this result implies that many genes involved in PGC specification such as Bmp2, Bmp4, Bmp8b, and Blimp1 [3-5, 15, 16, 243-247] and genes required for PGC migration and proliferation to the hindgut such as SCF, Kit, nanos3, and Hif-2a [26, 33, 216, 217, 248, 249] are working normally in *Ft* mutant embryos during early PGC development. However, many PGC failed to migrate into the genital ridge and proliferation failed once they reached the gonads. During this migration from the hindgut to the genital ridge, SDF-1/CXCR4 are important factors for PGC migration and proliferation. The expression pattern of SDF-1 was examined in the *Ft* mutant gonad, but no difference was detected between wild type and *Ft* mutant testes. Therefore,

we propose that the *Ft* locus includes new factors that control PGC migration and proliferation in gonads. More studies are needed to elucidate this mechanism.

3.5.2: Is expression of the *Ft* locus genes required for normal testis development?

Proper gene expression patterns are required for successful testis development. The role of the six genes of the *Ft* locus: *IrxB* cluster (*Irx3*, *Irx5*, and *Irx6*) and the other three genes (*Ftm*, *Fto*, and *Fts*) during testis development are unknown. However, this *Ft* locus is present in the testis beginning at stage of early embryonic testis formation, even though most genes show lower expression patterns in the embryonic testes than in embryonic ovaries. Thereafter, *Irx5*, *Ftm*, and *Fts* are up-regulated in the adult testis. Additionally, most of these genes are present primarily in somatic cells such as Sertoli, Leydig, peritubular myoid, or other interstitial cells. Therefore, we predicted that these genes may affect the function of somatic cells in addition to the interplay between somatic and germ cells during testis development. This hypothesis implies that the dysfunction of Sertoli cells and the failure of formation of normal spermatogonial cells in *Ft* mutant gonads are caused by a failure of interactions between germ cells and somatic cells.

Testis transplantation underneath the kidney capsule of castrated nude mice was used to circumvent early death of *Ft* mutant mice. Using this method, we found that *Ft* mutant gonads can develop normally until approximately the timing of birth (E12.5 gonads were transplanted underneath the kidney capsule and allowed to grow under the kidney capsule for 1 week ≈ ~birth), even though the *Ft* mutant testis has a small number of germ cells. This result confirms that male germ cells are not required for normal testis development [240]. Our immunostaining results indicate that *Ft* mutant testes showed normal formation of testis cords, normal germ cell development within testis cords, and normal Sertoli cell development at birth. Testes were then

transplanted for a total of two or three weeks to determine whether they could mature normally. After 3 weeks transplantation, *Ft* mutant testes showed low levels of MIS expression and disrupted Sertoli cell architecture. In addition, remaining germ cells failed to move toward the basement membrane suggesting a failure of connection between germ cells and abnormal Sertoli cells in the seminiferous epithelium. Together these cells normally create the spermatogonial stem cell niche, particularly for the A_{single} spermatogonia. In this niche, Sertoli cells produce the glial cell line-derived neurotrophic factor (GDNF) which affects maintenance of spermatogonial stem cell self-renewal [234, 250]. Thus, this first movement of germ cells toward seminiferous epithelium into seminiferous tubules is a very important process for normal spermatogenesis. Some of the *Ft* locus genes affect Sertoli cell differentiation, leading to failure of Sertoli cell maturation and disruption of the essential interaction with germ cells.

Our results indicate that the presence of the *Ft* locus is necessary for normal testis formation and this locus regulates directly or indirectly some important factors for normal testis development. Also, we predict that different combinations of genes may work at different times to cause the early germ cell phenotypes and the late Sertoli cell phenotypes. Functional studies of each *Ft* locus gene are necessary to ascertain the underlying defects that make up the phenotype of *Ft* mutant testes.

Figure 3.1: Expression patterns of the Ft locus genes from E12.5 to adult testes. Quantitative real time PCR results for *Irx3*, *Irx5*, *Irx6*, *Ftm*, *Fto*, and *Fts.* Fold change was calculated relative to transcript levels of the gonad at E12.0.

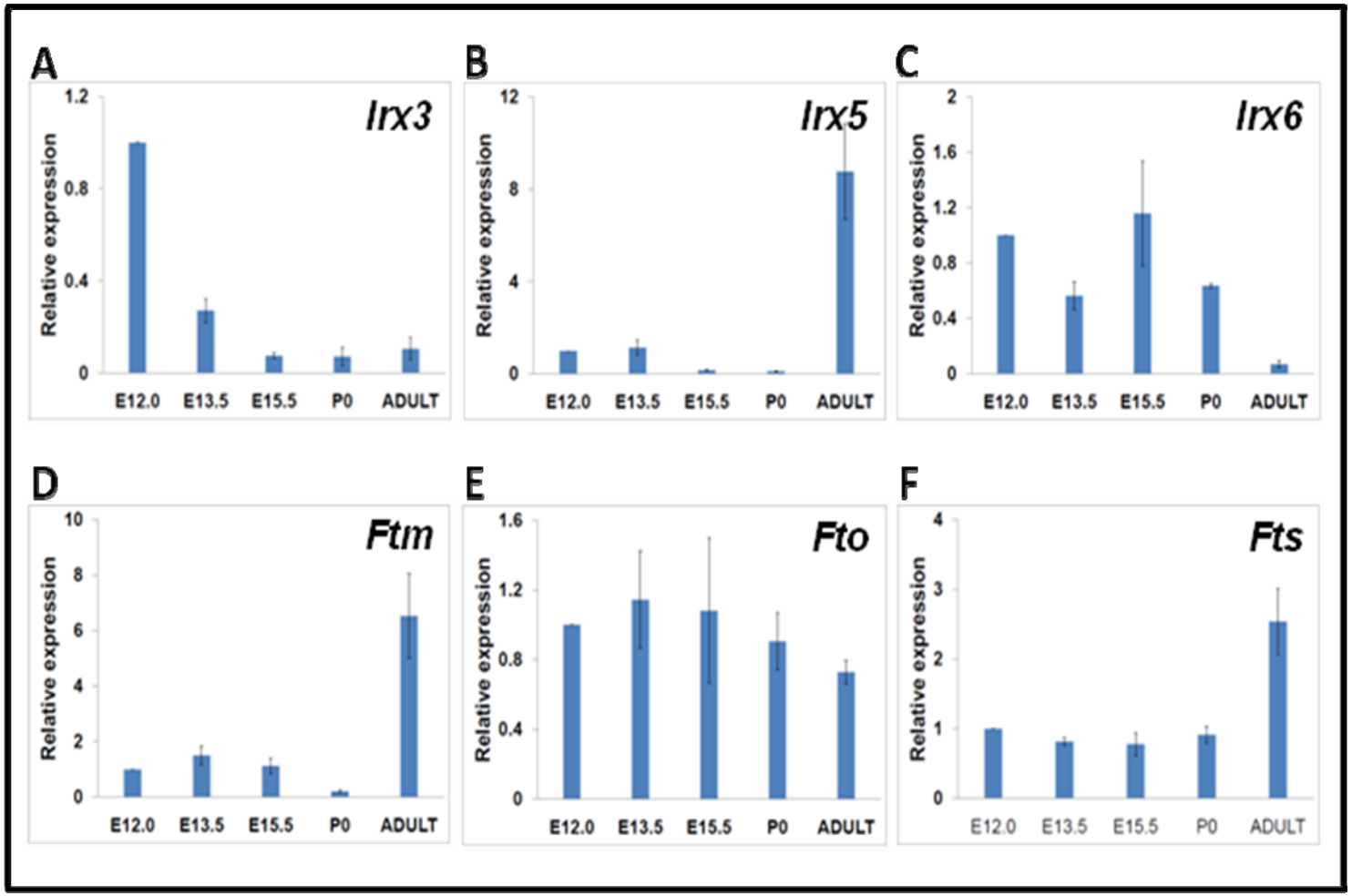


Figure 3.2: Quantitative PCR results showing expression of the Ft locus genes within male gonads harvested at E13.5 from embryos exposed to DMSO (Control, purple bars) or Busulfan (orange bars) treatment of pregnant females at E9.5. Single asterisk indicates p<2.43464E-13.

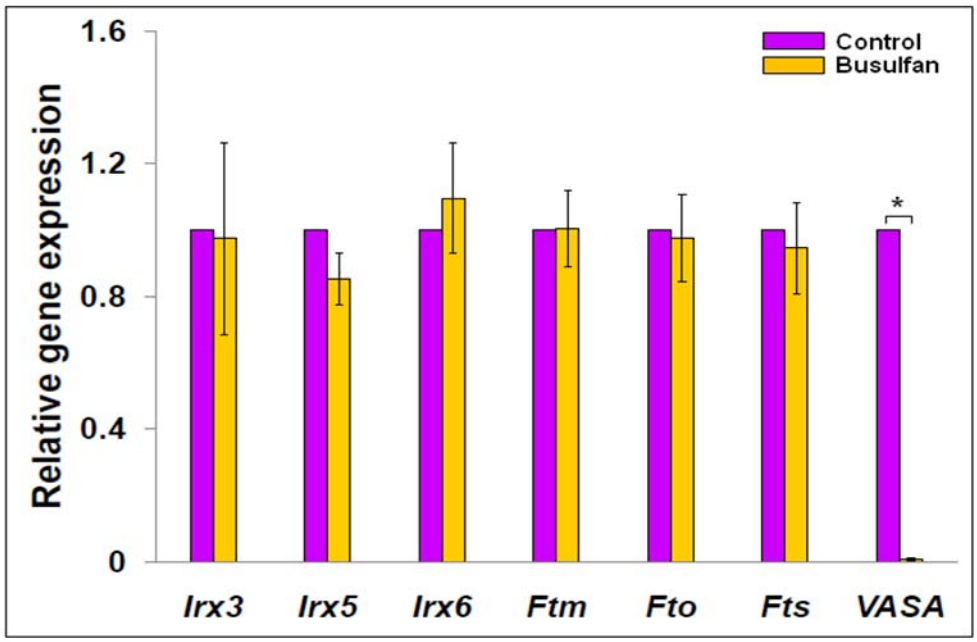


Figure 3.3: Ft mutant male gonads contain fewer germ cells. (A,B) Immunostaining for PECAM-1 protein in an E12.5 wild and Ft mutant ovary sections. (C) Comparison of the number of germ cells. Asterisk indicates p<0.001.

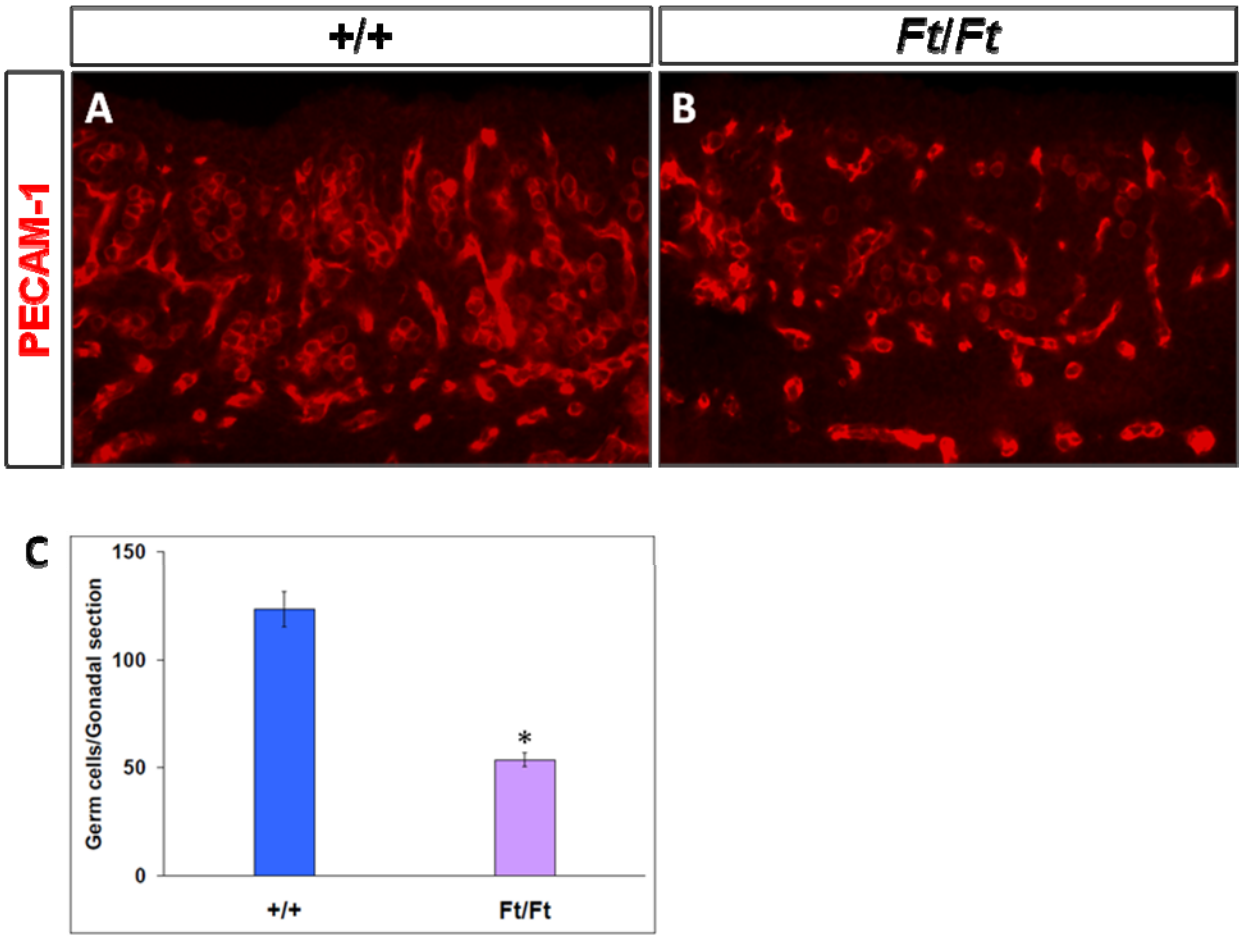
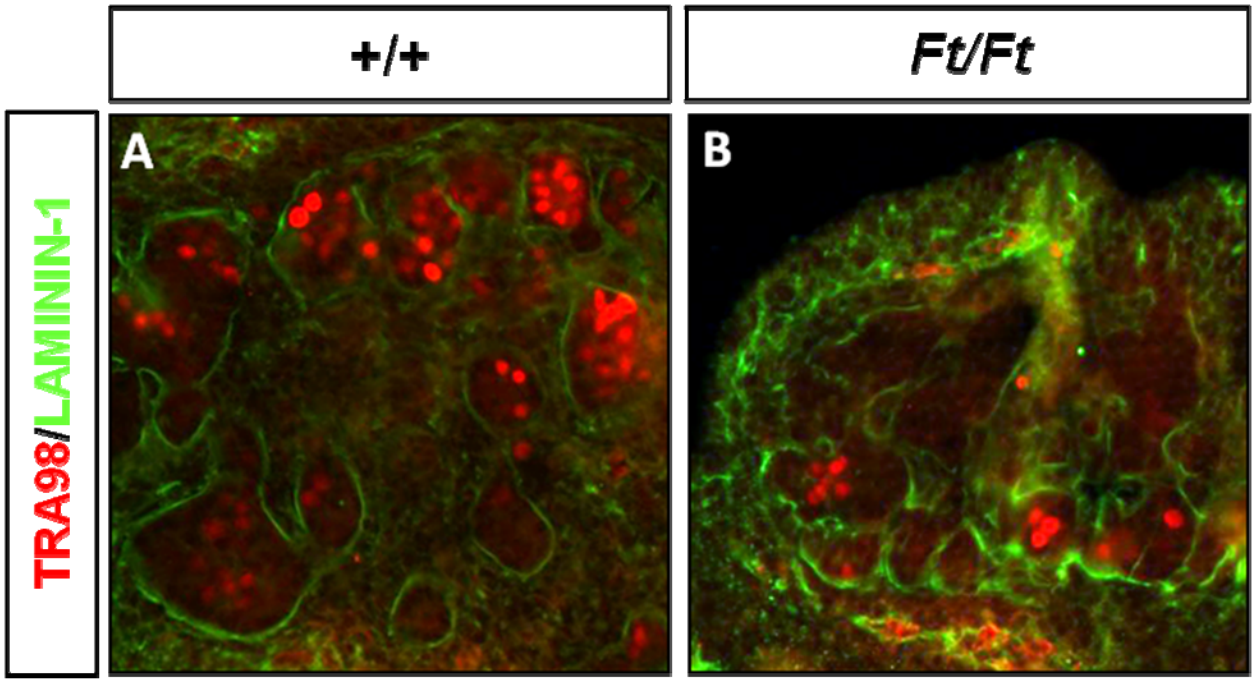


Figure 3.4: The reduced number of germ cells in E12.5 plus 2 days explants culture testes. (A,B) Double immunofluorescent staining for TRA98 protein (red) and MIS protein (green). (C) The number of germ cells in E12.5 plus two days explants culture. Asterisk represents statistical difference from the wild type ($p<0.01$).



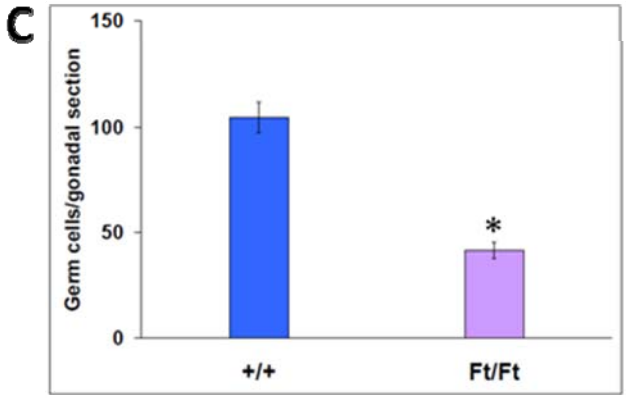


Figure 3.5: Vasa transcript levels are decreased in Ft mutant testes from E11.5 to E12.5 plus 2 days explant culture. Quantitative real time PCR was performed using primer for vasa and normalized to RNA of ribosomal subunit gene, 36B4 to obtain relative expression values. Relative expression of vasa was significantly decreased in Ft mutant testes (* $p<0.001$).

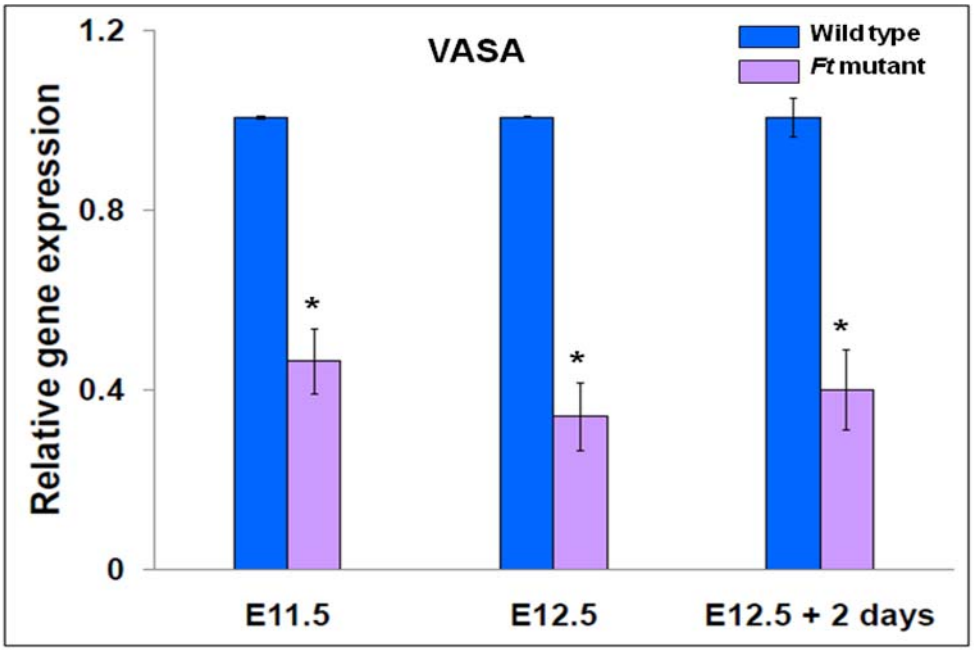
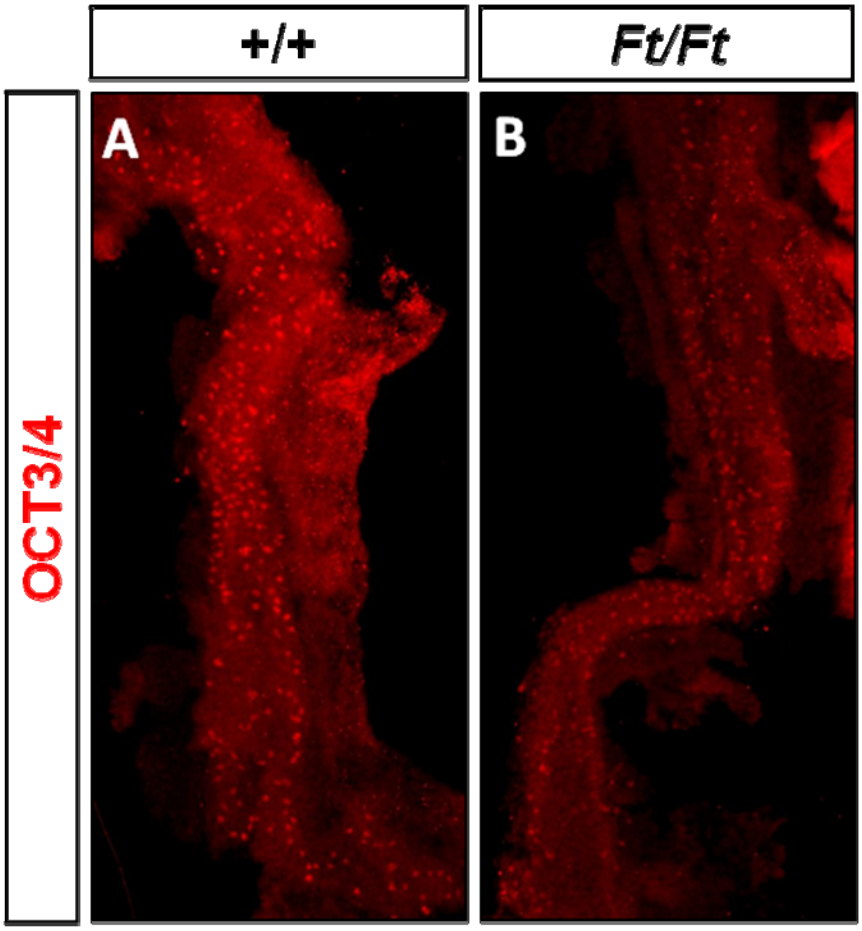


Figure 3.6: Whole mount immunostaining of primordial germ cells (PGC) in E9.5 embryos. (A) Immunofluorescent staining for OCT3/4 (red). (B) The number of PGC in wild and mutant type hindguts.



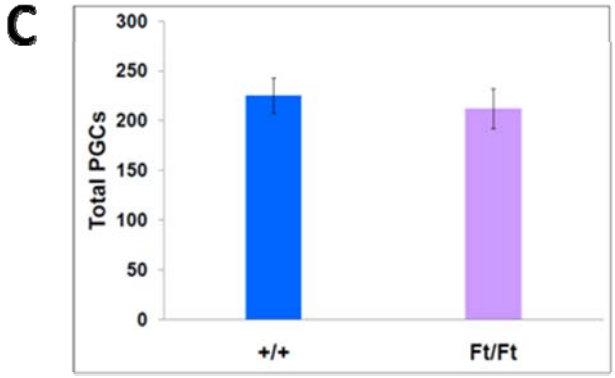


Figure 3.7: SDF-1 transcript levels in wild and *Ft* mutant testes.

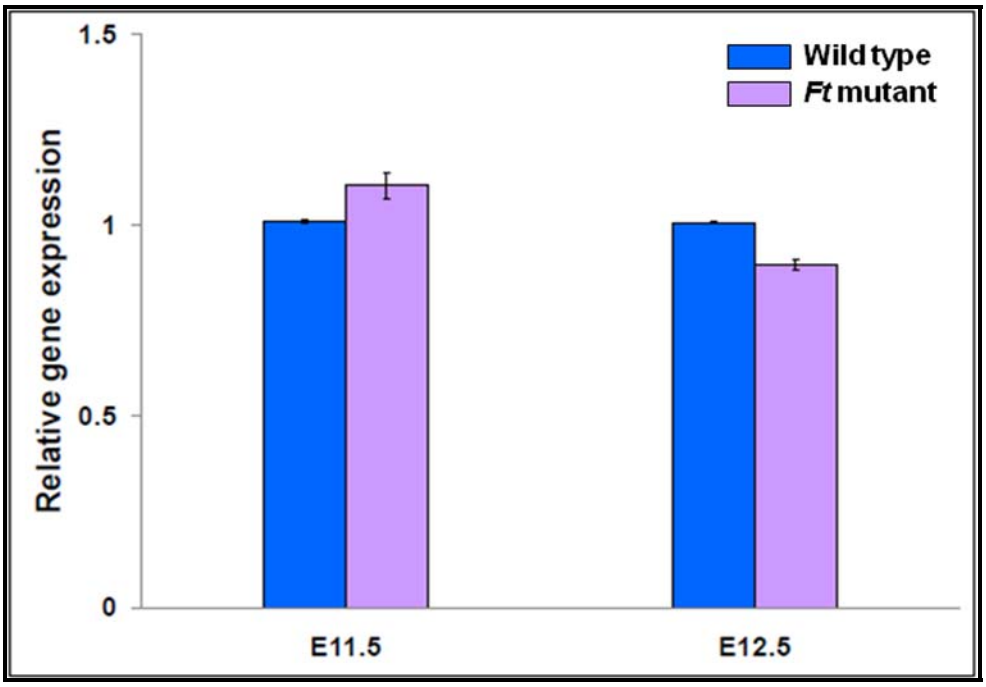


Figure 3.8: Germ cell proliferation is reduced in E12.5 *Ft* mutant testes. (A) Double immunofluorescence staining for PECAM-1 protein (green) and phosphorylated histone H3 protein (red) in E12.5 testis sections. (B) The percentage of mitotic germ cells. Asterisk represents statistical difference from the wild type ($p<0.01$). Arrows in (A) indicate mitotic germ cells.

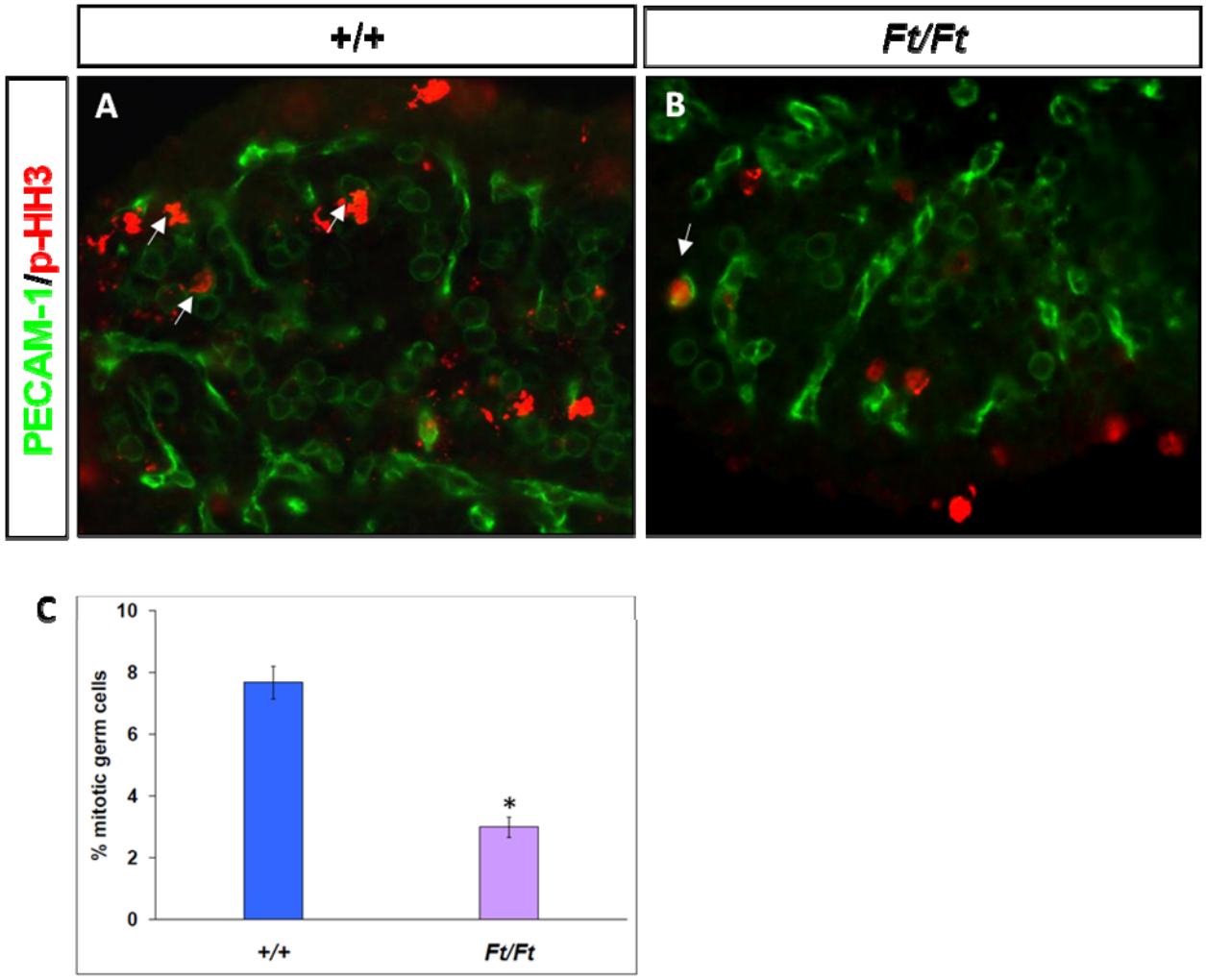


Figure 3.9: Germ cell proliferation is reduced in *Ft* mutant testes from E12.5 plus two days explants culture (A) Double immunofluorescent staining for TRA98 protein (red) and phosphorylated histone H3 protein (green) in testis sections. (B) The percentage of mitotic germ cells. Asterisk represents statistical difference from the wild type ($p<0.01$).

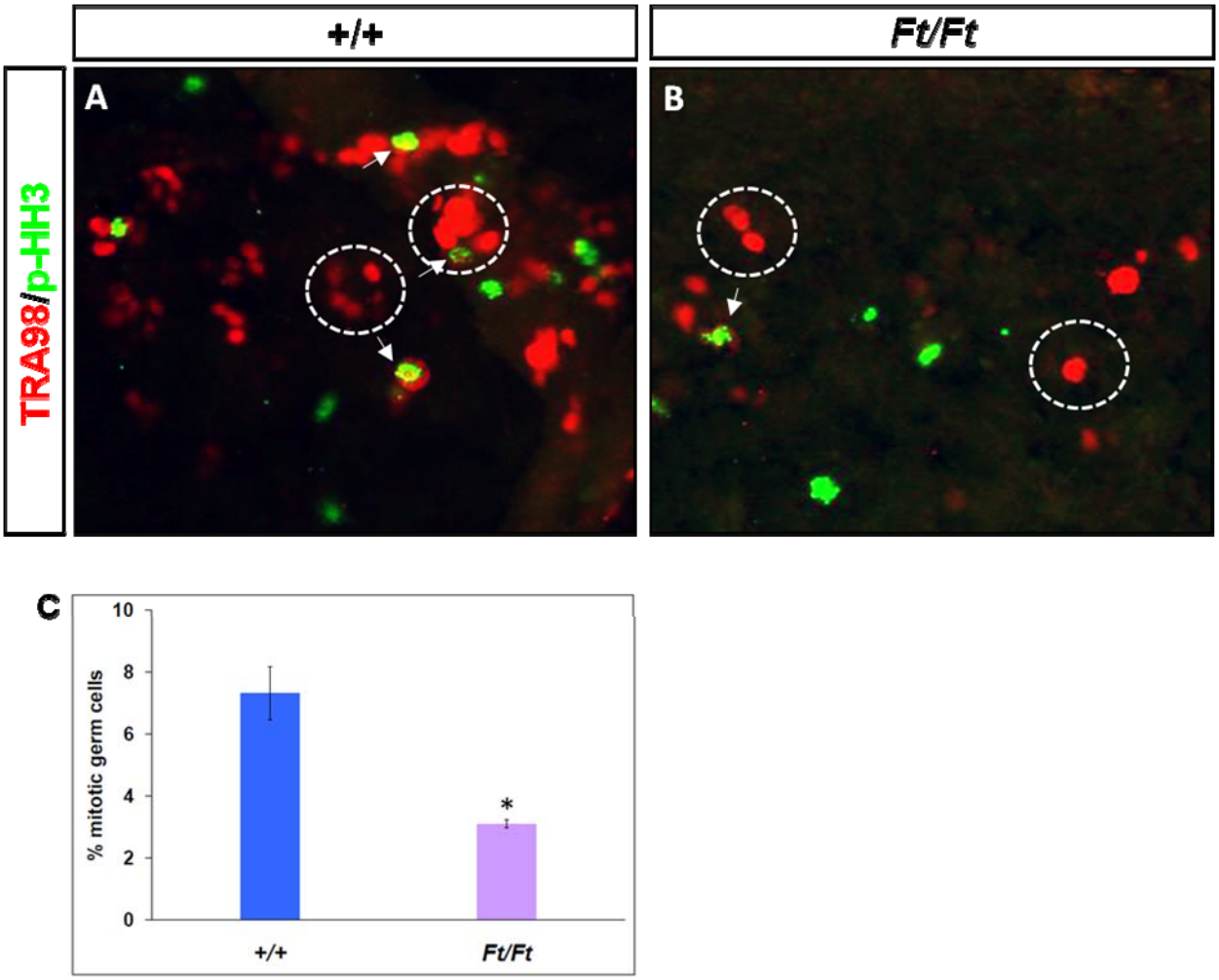


Figure 3.10: Bmp family member transcript levels in wild *vs*. *Ft* mutant testes.

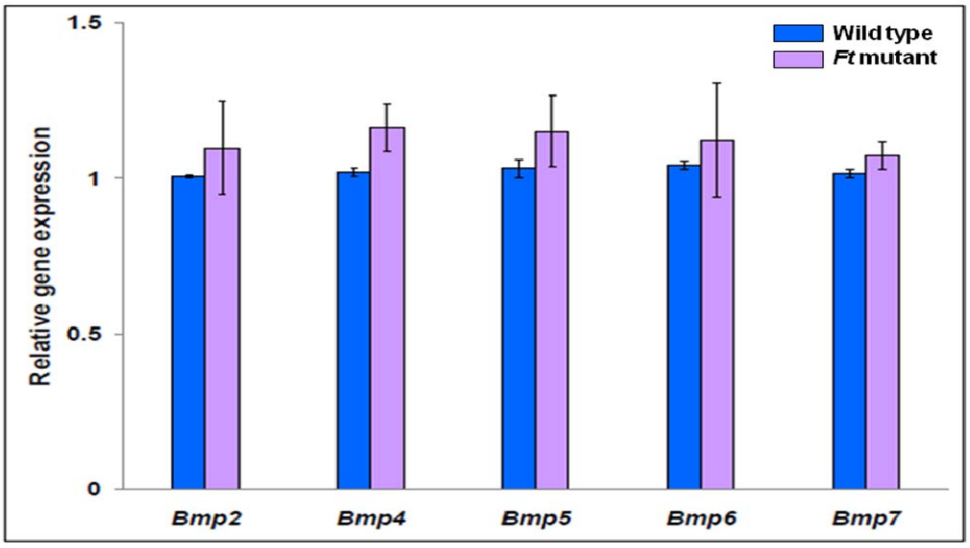


Figure 3.11: Testis cord histology in the wild and Ft mutant types. H&E-stained kidney grafted tissues 1week after testis transplantation. Arrows indicate germ cells and arrowheads indicate Sertoli cells. GC: germ cells; SC: Sertoli cells.

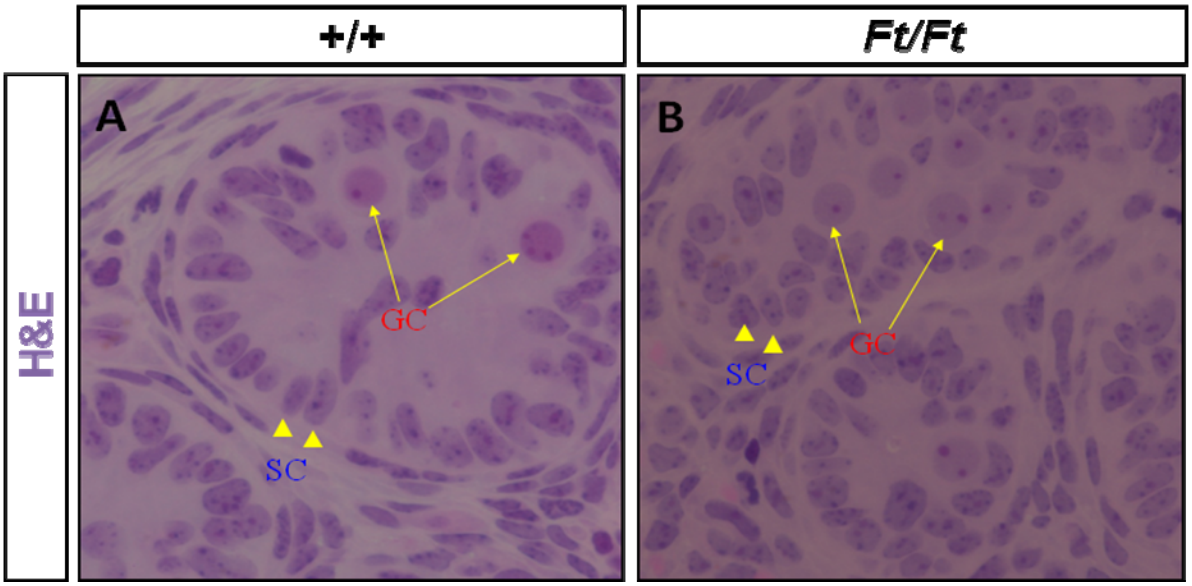


Figure 3.12: Normal testis formation 1 week after testis transplantation. (A, B) Double immunofluorescence staining for TRA98 protein (red) and LAMININ-1 protein (green). (C, D) Double immunofluorescence staining for MIS protein (red) and CYP17 protein (green).

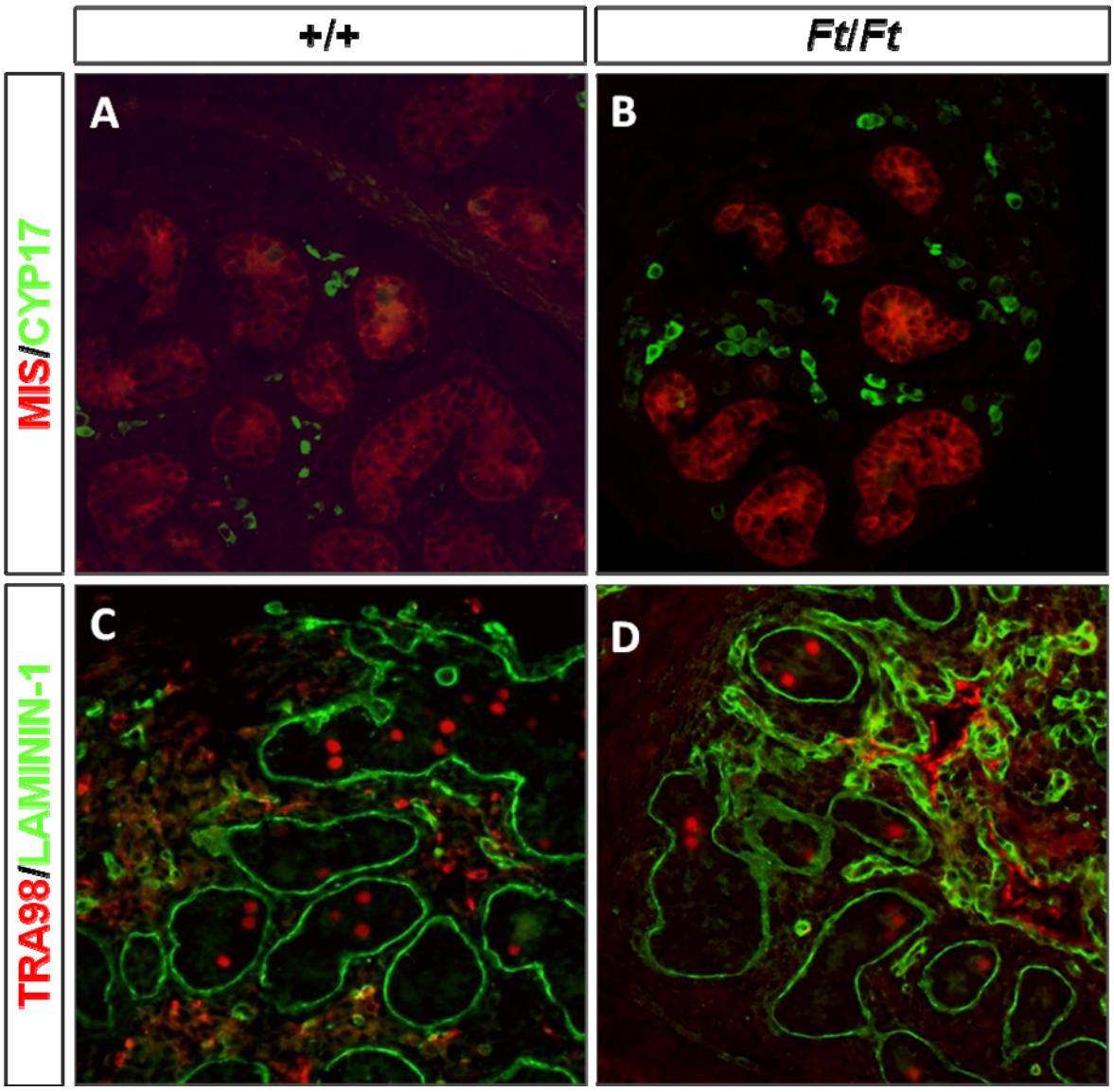


Figure 3.13: Testis cord morphology in grafted tissues 3 weeks after transplantation (under kidney for 1 week and under the dorsal skin for 2 weeks). H&E stained wild (a) and Ft mutant (b) testes. Arrows indicate a disruption of basal lamina.

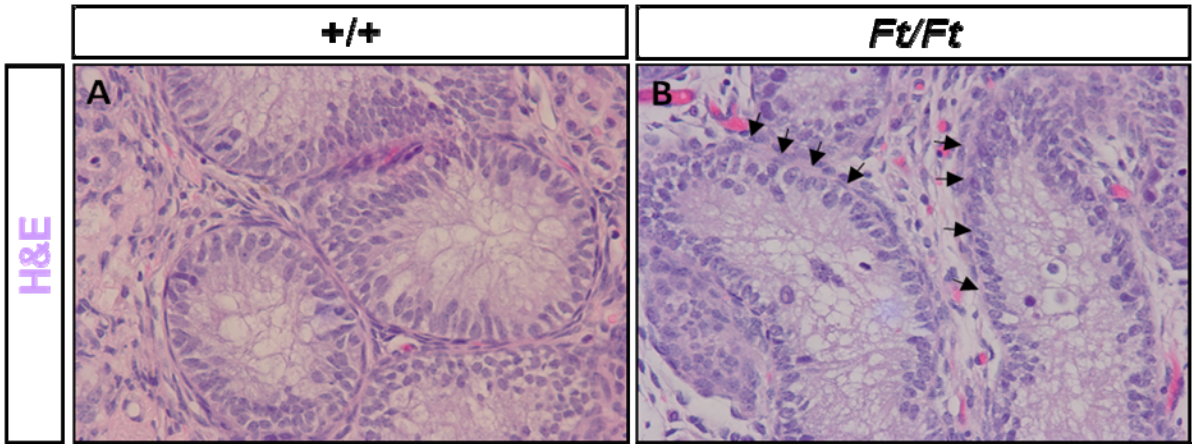
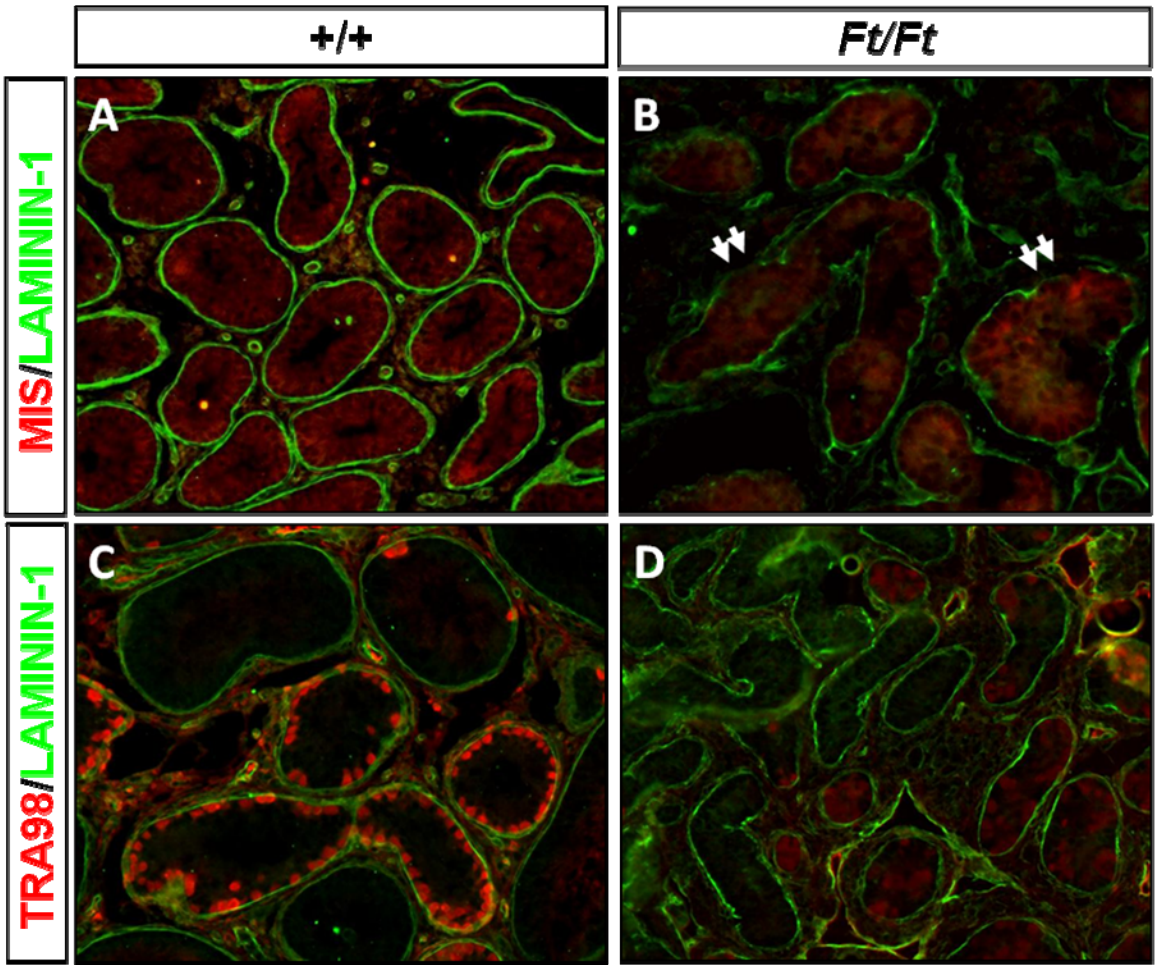


Figure 3.14: Failure of germ cell and Sertoli cell maturation and disruption of basal lamina in Ft mutant testes 3 week after transplantation. (A, B) Double immunofluorescence staining for TRA98 protein (red) and LAMININ-1 protein (green). (C, D) Double immunofluorescence staining for MIS protein (red) and LAMININ-1 protein (green). Arrows show disruption of basal lamina.



CHAPTER 4

THE ROLE OF IROQUOIS HOMEOBOX GENES 3 AND 5 DURING OVARY AND TESTIS DEVELOPMENT

4.1: ABSTRACT

Fused toes (*Ft*) mutant mice, which are missing the *IrxB* cluster, *Irx3*, *Irx5*, and *Irx6*, and *Ftm*, *Fto* and *Fts*, revealed many abnormalities during ovarian and/or testis developments: 1) Failure of primordial germ cell (PGC) migration and reduced germ cell proliferation in both male and female gonads; 2) Disruption of normal follicle formation in female gonads; 3) Disruption of Sertoli cell development in male gonads. To begin to understand contributions of individual genes within the Ft locus to the gonadal phenotypes, we obtained *Irx3* and *Irx5* mutant mice. The Iroquois homeobox (*Irx*) genes play fundamental roles in many developmental processes, but, at this time, the functions of *Irx3* and *Irx5* are unknown during male and female gonad development. In Chapters 2 and 3, we developed some ideas about the expression patterns of these two genes during male and female gonadal development. First, these genes are present in both gonads from early embryonic gonadal development. Second, both *Irx3* and *Irx5* genes are highly expressed in female gonads. Third, these two genes are expressed in somatic cells. Last, these two genes may compensate for each other. Based on these findings, we hypothesized that *Irx3* and *Irx5* are necessary for normal ovarian development but not required for normal testis development. Conventional or conditional *Irx3* and *Irx5* double knockout mice were used to investigate ovary and testis development.

Our preliminary results indicate that *Irx3* and *Irx5* genes are not required for early germ cell migration and proliferation. However, by birth (P0) these two genes are required for normal

ovary formation but are not necessary for testis development indicating a dimorphic functionality between the male and female gonad development.

4.2: INTRODUCTION

Many homeobox genes such as Lhx1, Lhx9, Emx2, Pbx1 and Hoxa11, Hoxa13, and Hoxd13 [43, 251-257] are known as important factors for early gonad development, other homeobox genes such as Nobox, Lhx8, Sprm1, Rhox5, Hox, and Pbx4 [92, 258-262] are important factors for oogenesis and spermatogenesis. In a previous study, I proposed that Iroquois homeobox (*Irx*) genes like other homeobox genes are also one of the important candidate genes for gonad development. The six mammalian *Irx* genes are organized into two clusters: *IrxA* and *IrxB* that are located on different chromosomes. In the mouse, *Irx1*, *Irx2*, and *Irx4* are located on chromosome 13 to form the *IrxA* cluster, whereas *Irx3*, *Irx5*, and *Irx6* are located on chromosome 8 to form the *IrxB* cluster [263]. Particularly, *Irx3* and *Irx5* of the *IrxB* gene cluster are expressed in both male and female gonads from an early stage of gonad development but other *Irx* genes are not [263].

In Chapters 2 and 3, I examined the function of six genes, including *IrxB* cluster genes, during ovary and testis development using the *Ft* mutant mice. My results suggested that these six genes were necessary for normal ovary and testis development. The *Ft* mutant ovary has severe abnormalities with regard to germ cell proliferation and follicle formation. The *Ft* testis has similar problems with germ cells and abnormal Sertoli cell differentiation. In addition, the expression patterns of these two genes (*Irx3* and *Irx5*) showed dimorphic expression between male and female gonads, in which they are highly expressed in female gonads during embryonic development. Based on these results, we hypothesized that *IrxB* cluster genes contribute to the

abnormalities that are unique to female gonads in the Ft mutant mouse. In Chapter 4, I used mice harboring either conventional or conditional double KO for *Irx3* and *5* genes. *Irx3* or *Irx5* single KO mice have normal gonad development in both the male and female and are viable and fertile. However, $Irx3^{-/-}$;$Irx5^{-/-}$ die early around E14.5 [Personal communication with Dr. Hui, University of Toronto]. From these results, I expect that these two genes compensate for each other during gonad development, so I generated conditional double mutant mice using the Cre/loxP system with $SF\text{-}1^{Cre}$ to circumvent early death of conventional double knockout mice. Preliminary data regarding the function of *Irx3* and *Irx5* genes during male and female gonad development is described here.

4.3: MATERIALS AND METHODS

4.3.1: Generation of global double knockout mice

$Irx3^{+/-}$;$Irx5^{+/G}$ double heterozygote mice (kindly provided by Dr. Chi-chung Hui, University of Toronto) (Fig. 4.1) were intercrossed to produce $Irx3^{+/+}$;$Irx5^{+/+}$, $Irx3^{+/-}$;$Irx5^{+/G}$, and $Irx3^{-/-}$;$Irx5^{G/G}$ embryos.

4.3.2: Generation of conditional double Knockout (c-dKO) mice

The standard Cre/loxP recombination system is used to produce c-dKO mice [264, 265]. Mice were crossed to SF-$1^{cre/+}$ mice (kindly provided by Dr. Keith Parker, University of Texas Southwestern Medical Center) [266]. The resulting $SF\text{-}1^{cre/+}$;$Irx3^{+/-}$;$Irx5^{+/G}$ mice were mated to $Irx3^{fl/+}$;$Irx5^{+/G}$ mice (kindly provided by Dr. Chi-chung Hui, University of Toronto) . Ultimately, $SF1^{cre/+}$;$Irx3^{fl/-}$;$Irx5^{G/G}$ mice were used to assess gonad development.

4.3.3: Genotyping

For breed stock, tail tissue samples were collected from 18-21 day old pups. For dissected embryos, tail tissue was collected. In both instances, tissues were digested in 200μL of buffer at 57℃ until completely dissolved. Following digestion, they were incubated at 95℃ for 30 minutes. Finally, the samples were centrifuged for 3 minutes at 13,000 x rpm at room temperature. Genotyping was accomplished via standard PCR.

4.3.4: RNA extraction and real-time PCR

RNA was prepared from pooled individual gonads (separated from mesonephros) from E13.5 gonads using the RNeasy Micro Kit (Qiagen). All quantitative real-time PCR assays were carried out using the SYBR Green I Kit (Applied Biosystems) as previously described [183]. The relative expression level for each sample was determined in the same run and was expressed as the ratio of the quantity of RNA of interest to that of a control RNA (36B4). Gene specific primers were designed using Primer Express; primer sequences are shown below.

4.3.5: Immunohistochemistry

Ovaries and testes were fixed in 4% paraformaldehyde in PBS at 4℃ overnight and then rinsed three times in PBS for 5 min each. Then samples were put through a sucrose gradient (10%, 15% and 20%) followed by incubation in 1:1 20% Sucrose and OCT-freezing media (Tissue-Tek) at 4℃ overnight. The samples were embedded in a 1:3 20% sucrose and OCT cocktail and cut into 10 μm frozen sections. Primary antibodies were as follows: goat anti-MIS (1:250; Santa Cruz Biotechnology), mouse anti-Tra98 (1:250; Cosmo Bio), goat anti-FOXL2 (1:250; Everest Biotech), and rabbit anti-LAMININ (1:250; Sigma-Aldrich). All secondary

antibodies (FITC or Rhodamine-conjugated donkey anti-rabbit, goat, and mouse antibodies) were obtained from Jackson Laboratory and used at a dilution of 1:300. Images were collected on a Nikon E600 microscope and processed using Adobe Photoshop.

4.3.6: Histology

Samples were fixed with 4% paraformaldehye (PFA) in PBS overnight at 4℃, processed through graded ethanol series, embedded in paraffin, sectioned to 5㎛, deparaffinized, rehydrated and stained with hematoxylin and eosin. Slides were mounted using Permount.

4.3.7: Statistical analysis

Statistical differences were determined using two-tailed Student's t test comparisons.

Table 4.1: PCR primers for real-time PCR (written 5' to 3')

Gene	Forward primer	Reverse primer
Irx3	CGCCTCAAGAAGGAGAACAAGA	CGCTCGCTCCCATAAGCAT
Irx5	GGCTACAACTCGCACCTCCA	CCAAGGAACCTGCCATACCG
Irx6	AGCACATCCCAGTTTCTGGTGTCT	ACAGCAAAGAGTAGAGGCAGAGGT
Vasa	GAGATTGCCTTCAGTACCTATGTG	GTGCTTGCCCTGGTAATTCT
Sox9	AGTACCCGCATCTGCACAAC	TACTTGTAATCGGGGTGGTCT
Foxl2	GCAAGGGAGGCGGGACAACAC	GAACGGGAACTTGGCTATGATGT
36B4	CGACCTGGAAGTCCAACTAC	ATCTGCTGCATCTGCTTG

Table 4.2: PCR primers for genotyping PCR (written 5’ to 3’)

Gene	Primer sequences	
Irx3 floxed or wild-type (WT)	Irx3-UP25544 5’-CAA GAA GGG GTG ATG AGA GTC GCT CCC CG-3’ Irx3-RP25864 5’-GGA GAG GGA ACC ACG GCG AGA AAG GCC TA-3’	323 bp (WT allele) 439 bp (Floxed allele)
Irx3 null	Irx3-UP25544 5’-CAA GAA GGG GTG ATG AGA GTC GCT CCC CG-3’ Irx3-RP25864 5’-GGA GAG GGA ACC ACG GCG AGA AAG GCC TA-3’ PGK-RP 5’-CCG GG GAT GTG GAA TGT GTG CGA GGC CA-5’	323 bp (WT allele) 381 bp (Null allele)
Irx5 EGFP	Irx5-UP11565 5’-GGT CCC GAA GGG CCA GAA TCA GAA TTG GGG-3’ Irx5-RP11899 5’-GCA TTC TTC CGG TAC GCG GGG TCC CCA TA-3’ PGK-RP 5’-CCG GG GAT GTG GAA TGT GTG CGA GGC CA-5’	337 bp (WT allele) 413 bp (Null allele)
SF-1/Cre	5’-GAG TGA ACG AAC CTG GTC GAA ATC AGT GCG-3’ 5’-GCA TTA CCG GTC GAT GCA ACG AGT GAT GAG-3’	408 bp (Cre positive) No band (Cre negative)

4.4: RESULTS

4.4.1*:* Different Irx6 expressions between female and male gonads.

To investigate whether *Irx6* genes compensate for loss of *Irx3/5*, I performed quantitative real-time PCR using $Irx3^{-/-}$;$Irx5^{G/G}$ gonads at E13.5. Generally, *Irx6* gene is present at very low expression levels (practically non-existent) in male and female gonads (Fig. 2.1C). PCR results showed no difference in *Irx6* expression levels in female gonads suggesting that there is no compensation between *Irx3/5* and *Irx6* (Fig. 4.2). In male gonads, however, Irx6 gene showed significantly increased expression patterns (≈ 4-fold higher) in $Irx3^{-/-}$;$Irx5^{G/G}$ gonads suggesting that there is compensation between *Irx3/5* and *Irx6* (Fig. 4.2).

4.4.2: $Irx3^{-/-}$;$Irx5^{G/G}$ male and female gonads develop normally until E13.5

Previously, we found that deletion of the *Ft* locus genes results in reduced germ cell numbers and decreased germ cell proliferation in both E12.5 and in E12.5 plus 2 days in vitro cultured male and female gonads in Chapters 2 and 3. Here I compared germ cell numbers between $Irx3^{-/-}$;$Irx5^{G/G}$ and wild type gonads using immunofluorescent staining in addition to quantitative real-time PCR to detect Vasa gene expression levels. No differences were detected in either germ cell numbers (Fig. 4.3; Fig. 4.4) or the gene expression levels of Vasa (Fig. 4.5).

To assess somatic cell maturation, I performed immunofluorescent staining with Foxl2 in female gonads (Fig. 4.3A-B) and Sox9/or MIS in male gonads (Fig. 4.4A-D). Results show no difference between the wild and $Irx3^{-/-}$;$Irx5^{G/G}$ gonads (Fig. 4.3; Fig. 4.4). Taken together, I concluded that *Irx3* and *Irx5* are not necessary in early gonad development. Early markers and results from germ cell migration, germ cell proliferation, or somatic cell function were not different from control. These findings are consistent with the patterns of gene expression as both

genes increase until E13.5. These data also suggest that *Ftm* is more important for germ cell migration and proliferation during early gonad development than are the *IrxB* cluster genes in the *Ft* mutant gonads. Therefore, I should consider the *Ftm* as a new candidate for early germ cell development. However, the functions of this gene in germ cell development remain to be determined.

4.4.3: Changes in gene transcripts were detected in $Irx3^{-/-}$;$Irx5^{G/G}$ at E13.5

Even though no difference was observed by immunofluorescent staining of FOXL2 or SOX9 in E13.5 female and male gonads respectively, I found that the mRNA levels of Foxl2 was reduced in $Irx3^{-/-}$;$Irx5^{G/G}$ female gonads and the mRNA of Sox9 was increased in $Irx3^{-/-}$;$Irx5^{G/G}$ male gonads (Fig. 4.5). These data suggest that changes in mRNA levels may not cause a significant impact on gonad development in the short term but additional studies are required to determine long term affects.

4.4.4: Conditional double knockout of *Irx3/5* results in reduced number of germ cells and primordial follicles and abnormal granulosa cell organization in P0 ovaries, but does not affect P0 testis development.

The deletion of *Irx3/5* genes showed normal development in both the male and the female until E13.5, however, $Irx3^{-/-}$;$Irx5^{G/G}$ mice show early embryonic lethality. Thus, to investigate a later stage of ovary and testis formation, I generated a conditional double knockout mouse model. To create this mouse model, I utilized two transgenic mouse lines – one expressing cre recombinase under the control of the steroidogenic factor 1(*SF1*) promoter [266] and the second in which exon2 of *Irx3* has been flanked by loxP sites. In the resulting $SF1^{cre/+}$; $Irx3^{fl/-}$;$Irx5^{-/-}$

cdKO (*SF1*-Cre dKO) mouse models in which *Irx3/5* were removed specifically within somatic cells of the fetal female and male gonads. First, to investigate ovarian development, Double label in immunohistochemistry was performed with Tra98 and Foxl2 or Tra98 and Laminin-1. Results showed that *SF1*-Cre; $Irx3^{fl/-}$;$Irx5^{G/G}$ deleted ovaries harbored a lower number of germ cells and primordial follicles than those of wild type ovaries. In addition, mutant ovaries showed irregular expression of Foxl2 in some areas (Fig. 4.6A-D).

In the male, *Irx3* expression is maintained at a very low level throughout life. *Irx5* is also barely expressed in the developing testis before puberty but it increases dramatically in the adult testis. In support of these expression profiles, I did not detect any abnormalities in the *SF1*-Cre; $Irx3^{fl/-}$;$Irx5^{G/G}$ testes by P0 when I assessed germ cell marker Tra98, Sertoli cell marker MIS and base membrane marker Laminin-1 (Fig. 4.7A-D). Based on these data, I conclude that *Irx3* and *Irx5* are not required for normal testis development until P0.

4.5: DISCUSSION

My preliminary data show dimorphic developmental effects of *Irx3* and *Irx5* during early ovarian and testis development. At E13.5, both female and male gonads of $Irx3^{-/-}$;$Irx5^{G/G}$ mice were normal. However, by P0 stage, the ovaries revealed developmental abnormalities including fewer germ cells and decreased expression of Foxl2 in granulosa cells that are irregularly placed. Male gonads showed normal development. In female gonads, the expression patterns of *Irx3* and *Irx5* start to increase from E12.5 and are maintained until birth. In male gonads, both genes are present at very low levels until birth. Here I suggest two possible roles for these two genes during male and female gonad development. First, the level of *Irx3* and *Irx5* expression would be important. Both *Irx3* and *Irx5* genes were present in testes at very low levels, but the absence of

these genes did not affect testis development, for they are not functional during embryonic testis development. This result implies that the level of gene expression must attain a certain standard in order to play a functional role in gonadal development. Low levels of *Irx3* and *Irx5* cannot regulate other factors during gonad development. No abnormalities in the female gonads at E13.5 were revealed despite high expression levels of these genes. Therefore, second, I suggest that exposure time to high levels of *Irx3* and *Irx5* is also important for normal functioning. As I described above, *Irx3* and *Irx5* genes begin to be highly expressed in female gonads starting at E12.5. After this time, both *Irx3* and *Irx5* genes increase in their mRNA and protein expressions. When the *Irx3/5* genes are deleted from female gonads for a short period of time prior to E 13.5, the female gonads look normal. However, when the *Irx3* and *Irx5* were absent in female gonads during the entire time prior to birth, normal ovarian development is disrupted because of failure of pre-granulosa cell maturation. These results support the idea that the maintenance of the high level of *Irx3* and *Irx5* may be required for normal (pre-) granulosa cell maturation in order to form normal follicles.

Foxl2 is a very important factor for normal ovarian development. I also surmise that Foxl2 is one of the potential candidate genes for interactions with or regulation by *Irx3/5*. In Chapter 2 and here in Chapter 4, I found that the deletion of the *Ft* locus or *Irx3/5* caused a decrease of Foxl2 during follicle development. In addition, the deletion of *Irx3/5* resulted in a decreasing of Foxl2 mRNA expression by quantitative real-time PCR by E13.5, but immunostaining intensities were not sensitive enough to detect a change in protein expression. However, abnormal expression of Foxl2 protein was observed in *SF1*-Cre $Irx3^{fl/-}$;$Irx5^{G/G}$ ovaries at P0, therefore, I predict that direct or indirect interactions between *Irx3/5* and Foxl2 are present

during ovarian development and that these interactions are necessary for normal follicle or ovarian formations.

Last, direct comparison between the *Ft* mutant and the *SF1*-Cre Irx3$^{fl/-}$;Irx5$^{G/G}$ mice was difficult because *Ft* mutant mice die during the early embryonic stage. However, my data suggest that *Ft* mutant ovary and testis showed more severe abnormalities. First, the disruption of germ cell migration and proliferation were only observed in *Ft* mutant mice. Second, many germ cells and primordial follicles were observed in *SF1*-Cre Irx3$^{fl/-}$;Irx5$^{G/G}$ ovaries, and in addition Irx3$^{-/-}$;Irx5$^{G/G}$ gonads have many germ cells at E13.5. Normal testis formation was additionally observed in *SF1*-Cre Irx3$^{fl/-}$;Irx5$^{G/G}$ males. These data indicate that *SF1*-Cre Irx3$^{fl/-}$;Irx5$^{G/G}$ mice have more minor problems in the ovary and testis than the *Ft* mutant mice. Therefore, other genes such as *Ftm*, *Fto*, and *Fts* also have roles for normal gonad development.

From these preliminary findings, I conclude that the exposure time and level of *Irx3* and *Irx5* are necessary for normal ovarian development, and the *Irx3/5* genes are also potential transcription factors for controlling the expression of Foxl2 during ovarian development, however, we need more functional studies to solidify these results.

Figure 4.1: Strategy for preparing the Irx5/EGFP Vector and generating transgenic mice. (Provided by Dr. Chi-chung Hui, University of Toronto)

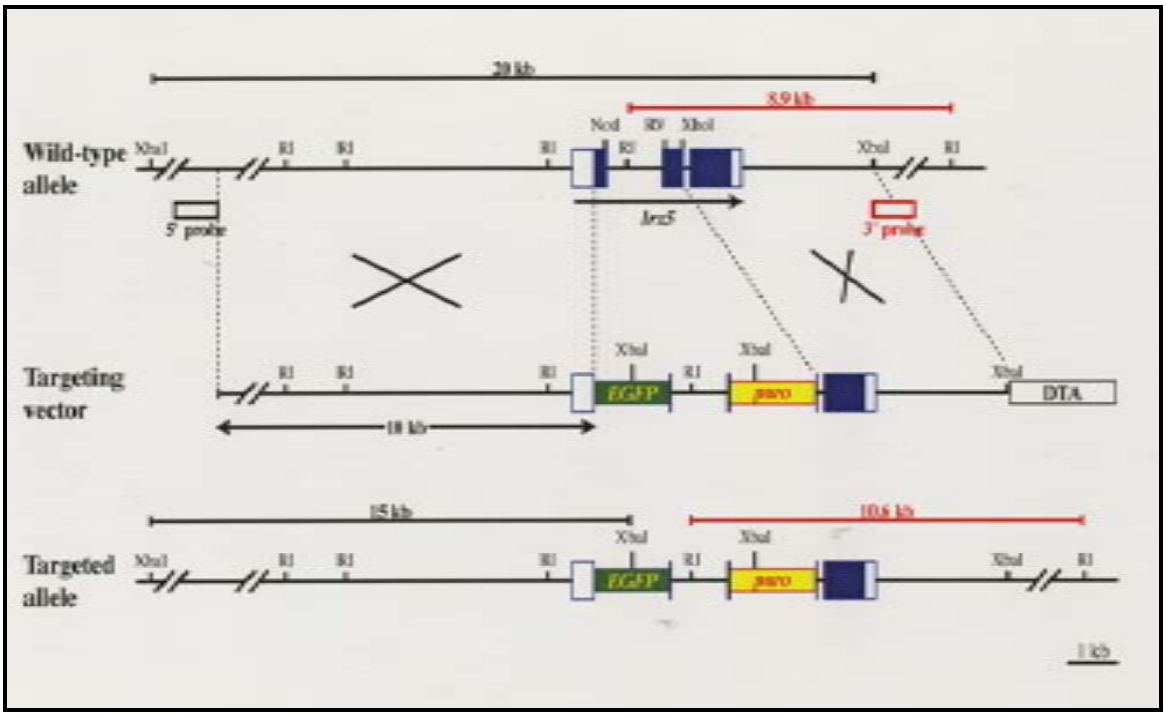


Figure 4.2: Quantitative real time PCR results for *Irx6* in female and male gonads from Irx3/5 double knockout mice. Asterisk indicates p<0.0001.

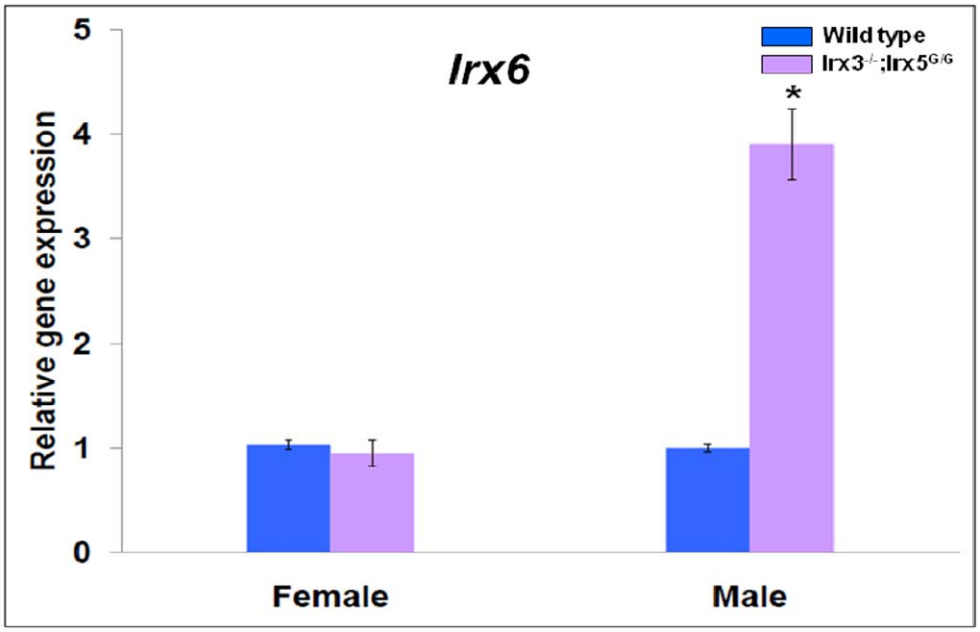


Figure 4.3: Normal ovarian development from the $Irx3^{-/-}$;$Irx5^{G/G}$ mouse model. Double immunofluorescent staining for TRA98 protein (red) and FOXL2 protein (green) from an E13.5 control (A) or double knockout section (B).

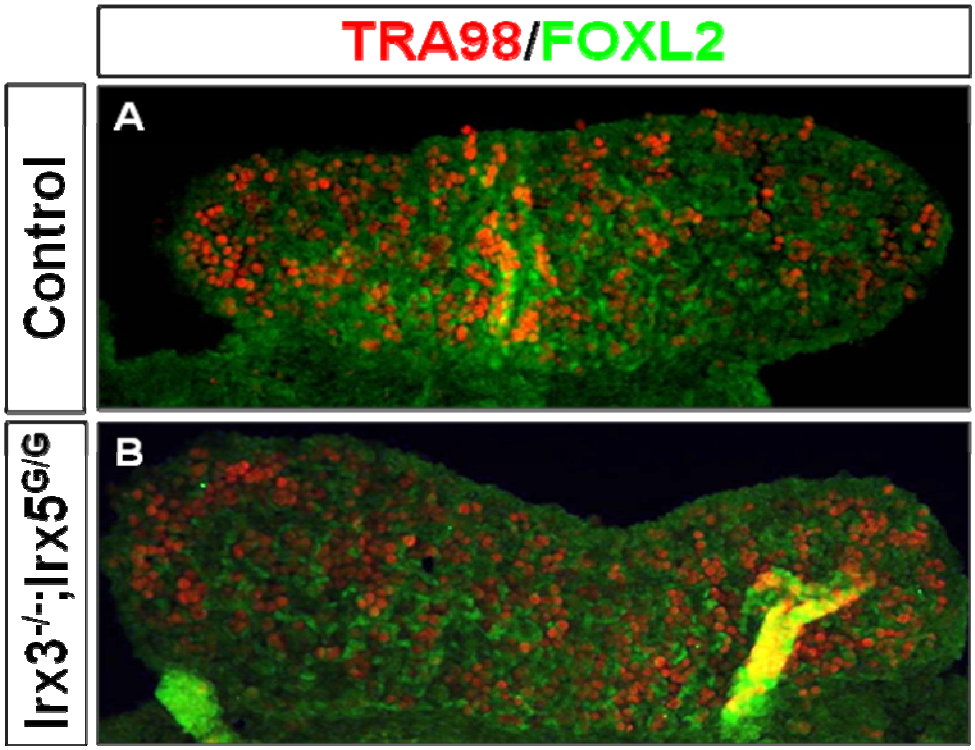


Figure 4.4: Normal testis development from the $Irx3^{-/-}$;$Irx5^{G/G}$ mouse model. (A, B) Double immunofluorescent staining for TRA98 protein (red) and MIS protein (green) from an E13.5 control (A) or double knockout section (B). (C, D) Double immunofluorescent staining for TRA98 protein (red) and SOX9 protein (green).

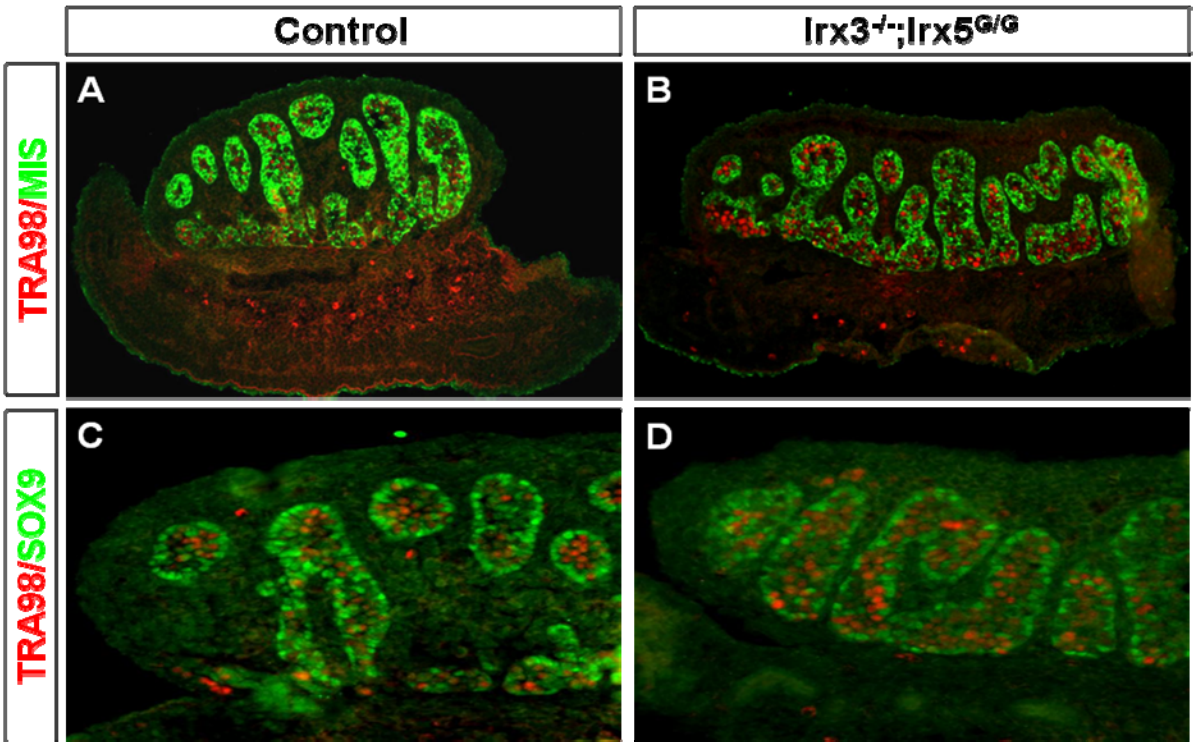


Figure 4.5: Quantitative real time PCR results for Vasa, Foxl2, and Sox9 in control (blue bars) and KO (purple bars) at E13.5. Asterisk indicates p<0.0007.

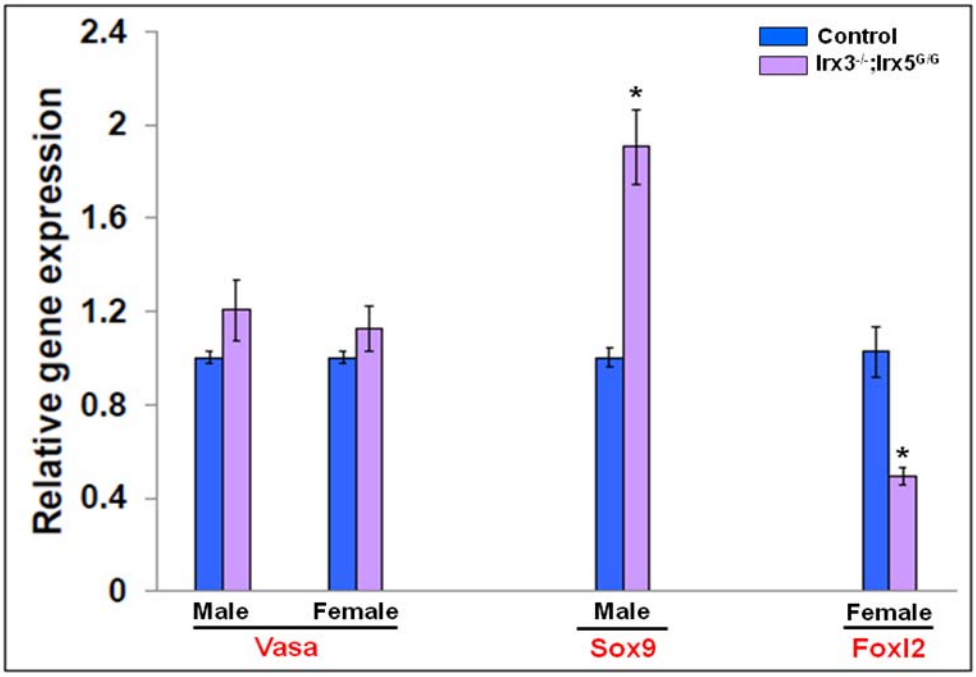


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Figure 4.6: A reduced number of germ cells and a decreased level of FOXL2 in Irx3/5 cdKO ovaries. (A, B) Double immunofluorescent staining for TRA98 protein (red) and FOXL2 protein (green) from an control (A) or double knockout ovary section (B). (C, D) Double immunofluorescent staining for TRA98 protein (red) and LAMININ-1 protein (green).

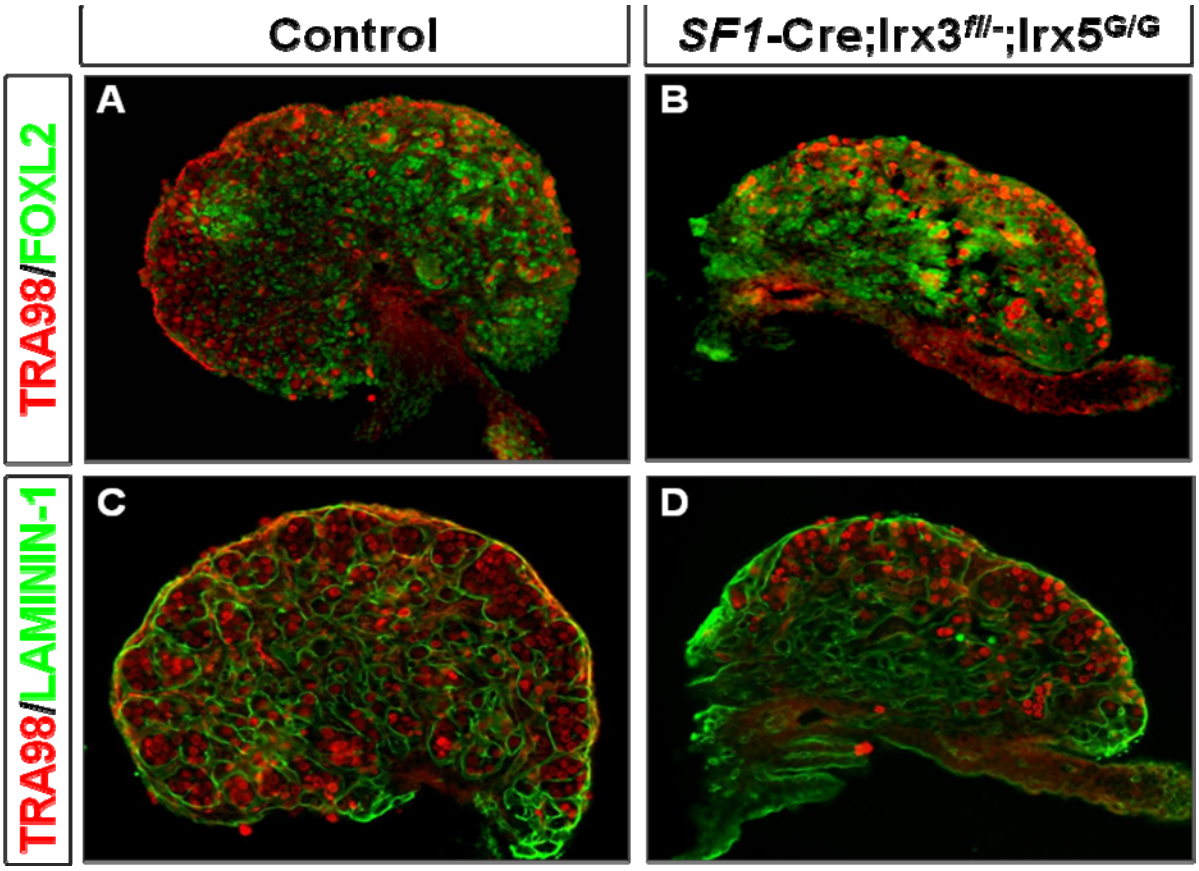
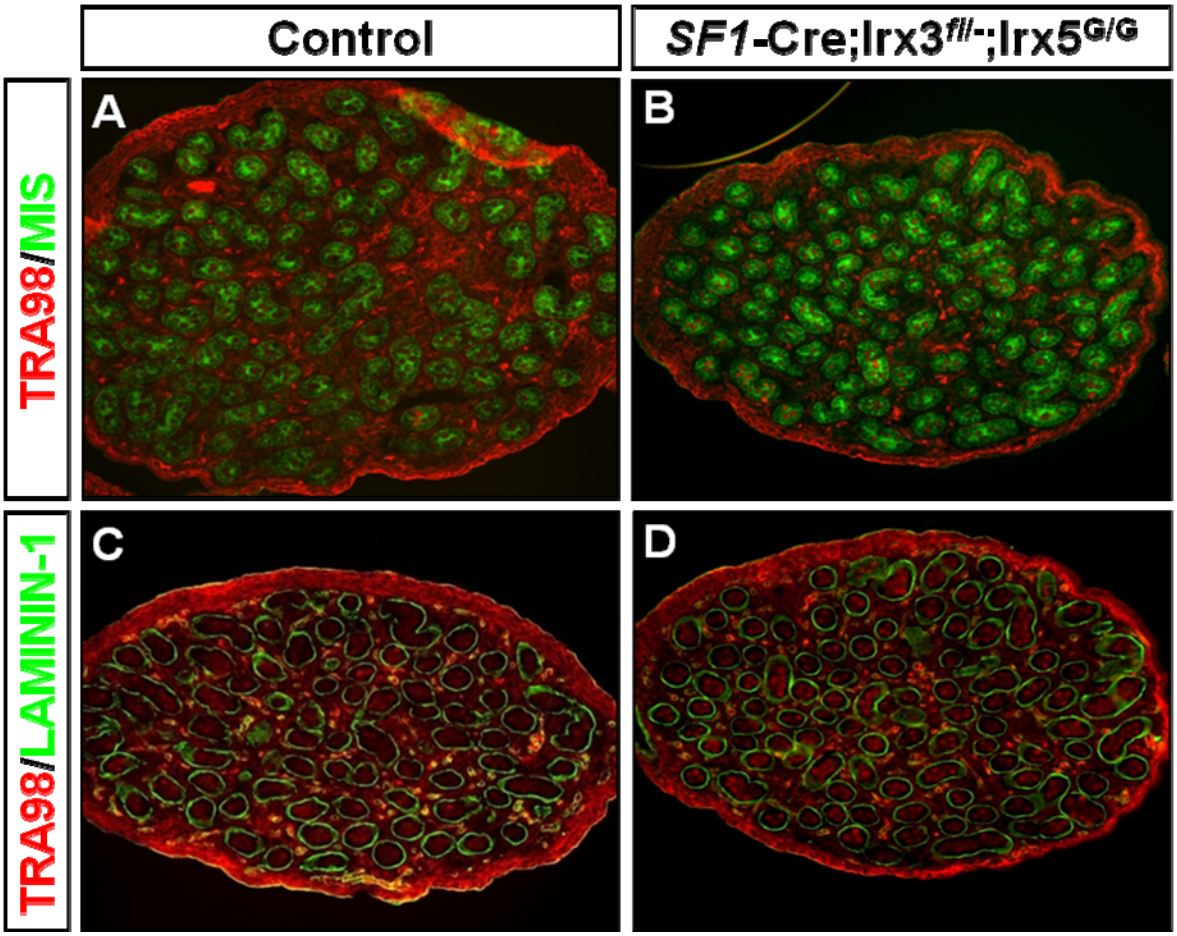


Figure 4.7: Normal testis development from Irx3/5 cdKO testes. (A, B) Double immunofluorescent staining for TRA98 protein (red) and MIS protein (green) from an control (A) or double knockout ovary section (B). (C, D) Double immunofluorescent staining for TRA98 protein (red) and LAMININ-1 protein (green).



CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

My doctoral research is the first study to investigate the function of the *Ft* locus genes during male and female gonadal development in mice. The *Ft* locus genes exhibit novel functions for ovarian and testis development. First, the *Ft* locus is important for PGC migration and proliferation. PGC in *Ft* mutant gonads fail to migrate into the genital ridge and the PGC in the genital ridge undergo reduced proliferation. These abnormalities during early gonadal development occur in both sexes. Second, in ovaries, the *Ft* locus affects follicle formation. *Ft* mutant ovaries showed a failure of follicle formation because of disrupted granulosa cell development. In testes, the *Ft* locus is important for normal Sertoli cell maturation. Severe abnormalities were observed in granulosa cells in the ovaries and their counterparts, the Sertoli cells in the testis. Therefore, these results support the idea that the *Ft* locus genes play important roles for somatic cell development during ovary and testis formation. In conclusion, the Ft locus genes are mostly present in the somatic cells of the ovary and testis, and the deletion of the entire *Ft* locus genes induces abolishment of communication between germ cells and somatic cells. Therefore, the ovary and testis lose their normal developmental pathway.

5.1: Conclusions

5.1.1: *Ftm, Fto,* and *Fts* genes are discovered as new important candidates for germ cell migration and proliferation during early gonad development

Ft mutant gonads showed a lower number of germ cells in the female and the male during the stage of early gonad development in E11.5. I proved that *Ft* mutant germ cells fail in their migration, and their proliferation is reduced. Interestingly, these abnormalities were not observed

in the conventional *Irx3* and *Irx5* double knockout mice. These data, together with double knock out results, suggest that at least one of three genes *Ftm*, *Fto*, and *Fts* of the *Ft* locus have an important function for early germ cell development in mice.

5.1.2: *IrxB* gene cluster is required for normal follicle development

Here we suggest that the *Ft* locus genes are necessary for normal follicle formation. The deletion of the entire *Ft* locus induces a severe disruption of granulosa cell formation, which is essential for normal follicle development. In addition, the dysfunction of granulosa cell development results in the abolishment of the interactions between granulosa cells and germ cells. Therefore, the *Ft* mutant follicles are blocked at the stage of primary follicles. *Irx3/5* double knockout ovaries resulted in a lower number of germ cells and an irregular positioning of granulosa cells. However, less severe abnormalities were observed in the conditional *Irx3/5* double knockout ovaries than in the *Ft* mutant ovaries. Therefore, it is possible that *Ftm*, *Fto*, and *Fts* are involved in and function during ovarian development such as follicle formation.

5.1.3: *Ft* locus is crucial for normal testis development

The *Ft* mutant testis was normal during embryonic gonad development. By 2 weeks after birth, an abnormal phenotype was detected in Sertoli cells, which is an important transition time for Sertoli cell maturation. The *Ft* mutant testis results in a decrease in MIS expression and a disruption of Sertoli cell architecture. These may cause a failure of germ cell maturation through the abolishment of the interactions between Sertoli and germ cells. However, there is no difference in the conditional *Irx3/5* double knockout versus the wild type testis. Therefore, we conclude that *Irx3/5* are not functional during early testis development.

The studies described in this thesis have identified new factors essential for ovarian and testis development. However, our findings raise many questions to elucidate the exact mechanism of *Ft* locus genes during male and female gonad development.

5.2: Future Directions

5.2.1: Can the deletion of *Irx3/5* abolish follicle formation?

In chapter 4, we have proposed that conditional *Irx3/5* double knockout ovary shows a decrease in the number of germ cells, and the partial disruption of granulosa cell formation. The function of *Irx3/5* during follicle formation using additional mice at a later stage of development must be determined. We do not believe that *Irx3/5* knockout ovaries induce a total disruption of follicle formation, but we expect that the *Irx3/5* knockout ovary will suffer reduced follicle formation and early cessation of ovarian function.

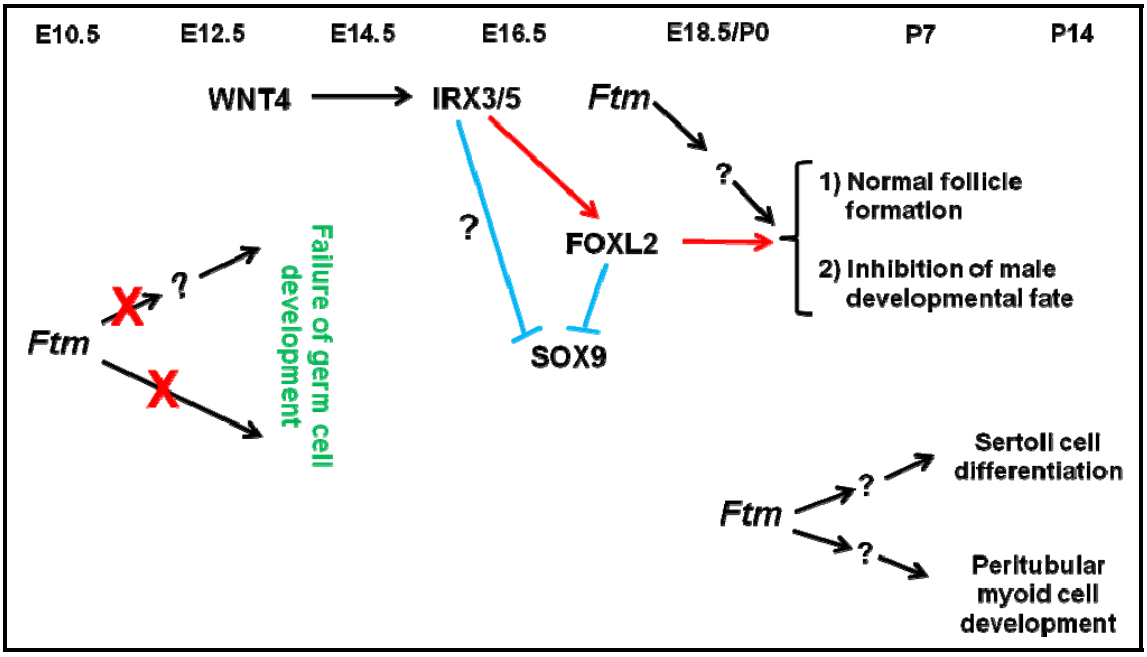
5.2.2: What is the separate function of individual *Ftm*, *Fto,* and *Fts* during testis or ovary development?

In my thesis, I suggest that these three genes are critical for male and female gonad development. However, further functional studies are necessary for a greater understanding of the individual functions of these genes during early male and female gonads, testis, and ovary development. One of the *Ft* locus genes, *Fto,* encodes a protein that can regulate obesity and diabetes [123, 157-162] through energy intake and metabolism regulation [163]. Recently, $Fto^{-/-}$ mice were discovered to be viable and fertile. Therefore, we do not think that *Fto* is directly involved in ovary or testis development. The functional roles of ovarian and testis development

in the other *Ft* locus gene, *Fts*, is also unknown. *Fts*$^{-/-}$ mice are considered viable, but nothing is known about their ovary and testis development. Therefore, an examination of the function of *Fts* during gonad development is necessary and required. The last *Ft* locus gene, *Ftm*, may be our most especially promising gene which anticipates some effect on testis and/or ovary formation. The *Ftm*$^{-/-}$ mouse model shares the most phenotypic changes with the *Ft* mutant mouse model, and because our preliminary data show that the *Ftm* has dimorphic gene expression patterns in developing female vs. male gonads, we expect that *Ftm* may be involved in many of the mechanisms for ovary or testis formation. The investigation into *Ftm*$^{-/-}$ mice will be valuable in determining the directly functional roles of the *Ftm* gene during ovarian and testis development.

Figure 5.1: A proposed model of *Irx3/5* and *Ftm* actions on male and female gonad development.

These studies were conducted for a greater understanding of the function of the Fused Toes locus during male and female gonad development. This locus includes six genes: *Irx3*, *Irx5*, and *Irx6* homeobox genes and three other genes: *Fto*, *Fts*, and *Ftm*. *Irx6* is not expressed in gonads [263] and does not compensate *Irx3/5* in gonads, and was therefore excluded, as was the *Fto* gene because it was reported to have no functional effect during gonad development. Additionally, the *Fts* knockout mice showed to be of a normal phenotype. I think that *Irx6*, *Fto*, and *Fts* are not necessary for normal testis and ovarian development, but that the remaining three genes: *Irx3/5* and *Ftm* are important candidate genes for normal testis and ovarian formation. Foremost, *Irx3/5* genes were considered the downstream of *Wnt4*, which is female gonad specific to early gonad development. *Wnt4* stimulates the expression of *Irx3/5* in female gonads. This increased expression of *Irx3/5* acts on the up-regulation of *Foxl2* which also induces *Sox9* depression in female gonads. However, it is still unknown whether *Irx3/5* directly regulates the expression of *Sox9*. The presence of *Irx3/5*, hereby, inhibits the male developmental fate in female gonads and maintains a proper environment for normal ovarian development. In male gonads, however, the low expression of *Wnt4* results in low *Irx3/5* expression as well as inactivation of *Foxl2*, therefore leading to a normal development of the testes.
Finally, little continues to be known about the function of *Ftm*, but may I suggest that the *Ftm* gene be considered as a candidate gene of primordial germ cell migration and proliferation during early gonad development in both sexes. *Ftm*, directly or indirectly, affects germ cell development, but we do not know yet exactly which function is involved. The *Ftm* may also affect normal ovarian and testis formation, but again the mechanism remains to be yet determined.



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CURRICULUM VITAE

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I. EDUCATION

2010 **Doctorate of Philosophy, Reproductive Biology**
Department of Animal Sciences
University of Illinois at Urbana-Champaign, Urbana, IL
Advisers: Janice S. Bahr, Ph.D. and Joan S. Jorgensen, Ph.D.
Dissertation: The role of the *Fused toes* locus containing *Iroquois* homeobox gene 3, 5, and 6 and *Ftm*, *Fto*, and *Fts* during ovary and testis development in mice

2002 **Master of Science, Animal sciences**
Chungbuk National University, South Korea
Adviser: Nam-Hyung Kim, Ph.D.
Thesis: Studies on the fertilization process in porcine oocytes following intracytoplasmic sperm injection

1998 **Bachelor of Science, Animal sciences**
Chungbuk National University, South Korea

II. RESEARCH EXPERIENCE

Ph.D. Graduate Research (2008 – Present)

***Advisor*:** Joan Jorgensen, DMV., Ph.D., University of Wisconsin – Madison

Accomplishments:

- Established the function of missing six genes during gonad development in Fused toes mutant mice
- Investigated function of *IrxB* gene cluster during gonad development in *Irx3* and *5* double KO mice
- Investigated primordial germ cell and early somatic cell development in mouse gonads

Ph.D. Graduate Research (2005 – 2008)

***Advisor*:** Joan Jorgensen, DMV., Ph.D., University of Illinois at Urbana-Champaign

Accomplishments:

- Established the function of missing six genes during gonad development in Fused toes mutant mice
- Investigate function of *IrxB* gene cluster during gonad development

Junior Researcher (2003 – 2005)

Department of Animal Biotechnology, National Institute of Animal Science, South Korea

Accomplishments:

- Investigated differentially expressed genes between normal and nuclear transferred embryos
- Performed discovery specific genes between embryos and uterus during porcine pregnancy

Internship Researcher (2000 – 2001)

EMBIX Biotechnology Inc., South Korea

Advisor: Hosup Shim, Ph.D., Dankook University (South Korea)

Accomplishments:

- Performed the production of transgenic pig embryos using ICSI

Master Research (2000-2002)

Advisor: Hyung Kim, Ph.D., Chungbuk National University (South Korea)

Accomplishments:

- Established Intracytoplasmic sperm injection in mice and pigs
- Investigated the mechanism of pronucleous formation during early fertilization

III. TECHNICAL EXPERIENCE

Molecular biology

Cloning, PCR, Real time PCR, In situ hybridization, Luciferase-reporter gene assay

Cell biology

Immunocytochemistry, Immunohistochemistry, Fluorescence microscopy, Confocal microscopy, Mammalian cell culture, Primary cell culture, Embryo culture, Organotypic culture of gonad tissues, Intracytoplasmic sperm injection (ICSI)

Experimental animal experiences

Experimental animal surgery: Mouse gonad dissection, Mouse castration and ovariectomy, Kidney capsule transplantation

IV. HONORS and AWARDS

2009	Lalor Foundation Merit Award by Society for the Study of Reproduction
2009	Larry Ewing Memorial Trainee Travel Fund by Society for the Study of Reproduction
2008	Travel grant award by the Graduate School, University of Illinois at Urbana-Champaign
2007-2008	Billie A. Field Fellowship from Department of Veterinary Biosciences, University of Illinois at Urbana-Champaign
2007	Travel grant award by Department of Veterinary Biosciences, University of Illinois at Urbana-Champaign
2000-2001	Scholarship from Graduate School, Chungbuk National University, South Korea
1996-1998	Chancellor's Scholarship from Chungbuk National University, South Korea
1992-1993	Chancellor's Scholarship from Chungbuk National University, South Korea

V. PUBLICATIONS

1. B Kim, Y Kim, and JS Jorgensen. Failure of germ cell proliferation and degeneration of follicles during ovarian development in fused toes mutant mice. (Manuscript in preparation)

2. B Kim, Y Kim, and JS Jorgensen. Reduced germ cell proliferation during early and disruption of Sertoli cell morphology during late testis development in Fused toes (*Ft*) mutant mice. (Manuscript in preparation)

3. Liying Gao, Y Kim, B Kim, Stacey Lofgren, Jennifer Schultz, Ann Nardulli, Leslie Heckert, and Joan Jorgensen. Two regions within the proximal SF1 promoter work together to drive somatic cell-specific activity in developing gonads of the female mouse. (submitted)

4. Yu-Jeong Jeong, Bong-Ki Kim, Xiang-Sun Cui and Nam-Hyung Kim. 2005. Haploidy influences *bak* and *bcl2* mRNA expression and increases of apoptosis in porcine embryos. Zygote. 13(1):17-21.

5. H. J. Chung, J. K. Park, B. K. Kim, Y. K. Lee, S. K. Im, H. H. Seong, S. J. Uhm, H. T. Lee, K. S. Chung, K. S. Min, J. H. Kim, N. Wakasugi and W. K. Chang. 2003. Differences in embryonic development in sensitive and resistant matings to pregnancy block stimuli in mice. Reproduction. 126:327-335.

6. Bong-Ki Kim**,** S.H. Jun**,** Y.J. Lee, X.S. Cui and N.H. Kim. 2003. Pronucleus formation, DNA synthesis and metaphase entry in porcine oocytes following intracytoplasmic injection of murine spermatozoa**.** Zygote. 11:261-270.

7. Bong-Ki Kim, Y.J. Lee, X.S. Cui and N.H. Kim. 2002. Chromatin and Microtubule organization in maturing and Pre-activated porcine oocytes following intracytoplasmic sperm injection**.** Zygote. 10:123-129.

8. Y.J. Lee, Bong-Ki Kim, X.S. Cui and N.H. Kim. 2002. Male pronuclear formation and sperm mitochodria in porcine oocytes following intracytoplasmic injection of pig or mouse sperm. Zygote. 10:117-122.

9. X.S. Cui, Bong-Ki. Kim, S.H. Jun, C.S. Park, N.H. Kim. 2002. Onset of pronuclear formation and DNA synthesis in porcine oocytes following intracytoplasmic injection of porcine or murine spermatozoa**.** Korea Journal of Animal Reproduction. 26(4):361-368.

VI. PRESENTATIONS

Oral presentations

1. B Kim, Y Kim, and JS Jorgensen. Abnormal female gonad development in *Ft* mutant mice. The 42nd Annual meeting SSR, 2009

2. B Kim, Y Kim, and JS Jorgensen. Abnormal female gonad development in Ft mutant mice. The 30th Annual minisymposium on reproductive biology, Northwestern University, 2009

Poster Presentations

1. B Kim, Y Kim, and JS Jorgensen. Abnormal development in female gonads of fused toes mutant mice. The 40th Annual meeting SSR, 2007.

2. B Kim, Y Kim, and JS Jorgensen. Apoptosis is absent in female gonads of Fused toes mutant mice during development. The 27th Annual minisymposium on reproductive biology, Northwestern University, 2006.

3. B Kim, Y Kim, and JS Jorgensen. Abnormal development in female gonads of Fused toes mutant mice. The 19th research symposium, UIUC, 2006.

4. Bongki Kim, and G Im. The major gene expression patterns in endometrial tissue of pigs during early gestation. Annual Conference of the International Embryo Transfer Society. Copenhagen, Denmark, 2005

5. H Chung and Bongki Kim. Expression of Oct-4 in the pregnancy of Korean native cattle. The 11th AAAP Animal Science Congress. Kualu Lumpur, Malaysia, 2004.

6. Bongki Kim. Expression patterns of TGF-beta1 and type I and type II of TGF-beta receptors in bovine embryos. Annual Conference of the International Embryo Transfer Society. Portland, Oregon, USA, 2004